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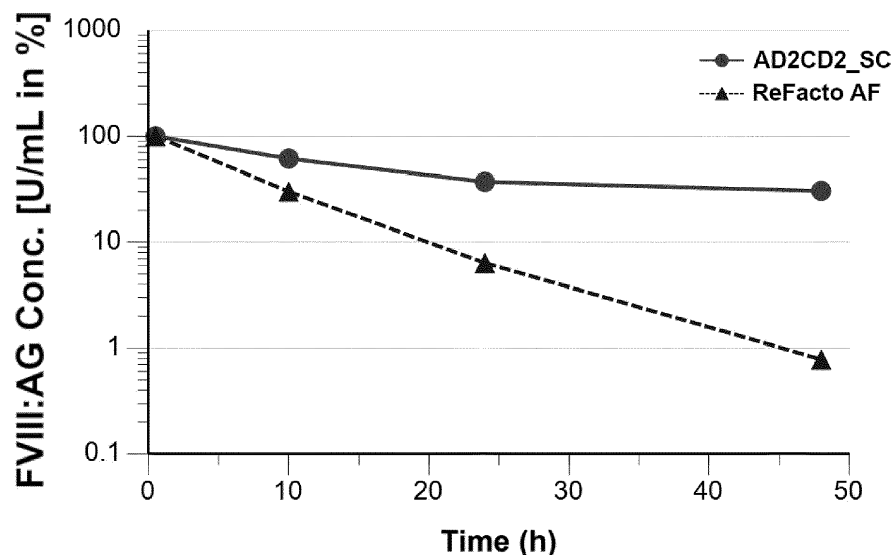
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(54) **FACTOR VIII PROTEIN WITH INCREASED HALF-LIFE**

(57) The present invention relates to recombinant coagulation factors, in particular, recombinant Factor VIII (FVIII) proteins having an increased half-life. They comprise a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion. If

the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion. The invention also relates to nucleic acids encoding the proteins of the invention, methods of producing them and pharmaceutical compositions comprising any of these, wherein the pharmaceutical composition preferably is for use in treatment of hemophilia A.

Fig. 5



## Description

**[0001]** The present invention relates to recombinant coagulation factors, in particular, recombinant Factor VIII (FVIII) proteins having an increased half-life. They comprise a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion. If the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion. The invention also relates to nucleic acids encoding the proteins of the invention, methods of producing them and pharmaceutical compositions comprising any of these, wherein the pharmaceutical composition preferably is for use in treatment of hemophilia A.

**[0002]** FVIII is an important co-factor in the coagulation cascade. Wildtype human FVIII is synthesized as a single chain consisting of 2351 amino acids and comprises three A domains (A1-A3), one B domain and two C domains (C1 and C2), interrupted by short acidic sequences (a1-a3). The first 19 amino acids are the signal sequence, which is cleaved by intracellular proteases, leading to a FVIII molecule of 2332 amino acids. The resulting domain structure is A1-a1-A2-a2-B-a3-A3-C1-C2. During post-translational modification, FVIII becomes glycosylated, sulfated and proteolytically processed. Sulfation is important for the extracellular interaction with different proteins, especially Thrombin and von Willebrand factor (vWF). It takes place on six tyrosines in the acidic regions a1, a2 and a3. Intracellular cleavage, by the serine protease furin, divides FVIII into a heavy chain (A1-a1-A2-a2-B) and a light chain (a3-A3-C1-C2). During this cleavage, parts of the B domain can be lost. Therefore, the light chain has a molecular weight of 80 kDa, whereas the heavy chain can be slightly heterogeneous, with a molecular weight around 210 kDa. The binding between heavy and light chain is not covalent, but mediated by the divalent metal ion  $\text{Cu}^{2+}$  between the A1 and A3 domain.

**[0003]** In the circulation, FVIII is bound to vWF via the a3, C1 and C2 domain, which protects FVIII from early activation as well as degradation.

**[0004]** Upon activation, FVIII is cleaved by Thrombin at three positions, leading to a heterotrimer and loss of the B domain (heterotrimeric FVIIIa). The heterotrimer forms a complex with the activated coagulation Factor IXa and coagulation Factor X, and the light chain binds to a phospholipid bilayer, e.g., the cell membrane of (activated) platelets.

**[0005]** Hemophilia A mainly is a genetic bleeding disorder linked to the X-chromosome, occurring in 1 of 5000 newborn males. However, Hemophilia A can also occur spontaneously due to an auto-immune response against FVIII. Patients with Hemophilia A suffer from longer bleeding durations, spontaneous and internal bleedings, affecting their everyday life.

**[0006]** Hemophilia A patients are generally treated by administration of FVIII. Depending on the severity of the disease (mild, moderate or severe), treatment is on demand or prophylactic. Therapeutic FVIII products are either purified from human plasma (pFVIII) or the products are produced recombinantly in cell culture (rFVIII).

**[0007]** During the development of recombinant FVIII molecules for therapy, B-domain deleted FVIII molecules have been designed, because the B-domain is not important for the functionality of FVIII in clotting. This predominantly leads to a reduction in size. One of the most common B-domain deleted FVIII product is ReFacto® or ReFacto AF® produced by Pfizer. This FVIII variant lacks 894 amino acids of the B domain.

**[0008]** One issue with regard to FVIII substitution therapies is the relatively low in vivo half-life of the plasma-derived or recombinant Factor VIII proteins. There have been different approaches for enhancing FVIII half-life reaching from PEGylation to albumin incorporation, single chain molecules and Fc fusion.

**[0009]** WO 2014/070953 A1 relates to a method of reducing or decreasing the bleeding rate of a hemophilia patient by administering a long acting Factor VIII polypeptide, wherein the long acting Factor VIII polypeptide may be a fusion of the Factor VIII polypeptide and a heterologous moiety, which is an FcRn binding partner and may comprise an Fc region.

**[0010]** WO 91/01743 A1 describes a process for extending the half-life of a biologically active protein or peptide in vivo by covalently coupling said protein or peptide to a polypeptide fragment capable of binding to a serum protein, especially to serum albumin. Preferably, the albumin-binding fragment derives from streptococcal protein G or staphylococcal protein A. The generated fusion protein binds to serum albumin in vivo, and benefits from its longer half-life, thereby increasing the net half-life of the fused therapeutically interesting protein or peptide. WO 2005/097202 A2 generally mentions fusion proteins combining a therapeutic protein and a plasma protein in a single polypeptide chain, wherein such fusion proteins may provide clinical benefits in requiring less frequent injection and higher levels of therapeutic protein in vivo.

**[0011]** WO 2009/016043 A2 discloses an albumin binding polypeptide. WO 2010/054699 A1 discloses a capture molecule for modulation of pharmacokinetics and/or pharmacodynamics of a target having a biological function in a mammal, wherein said molecule comprises at least one albumin binding moiety.

**[0012]** WO 2011/101284 A1 describes Factor VIII fusion proteins. Reference to albumin binding moieties is made. Fusion partners are presumed to delay in vivo clearance of FVIII by interaction with serum albumin. Albumin binding polypeptides, such as the ABD1 polypeptide, are mentioned as examples for fusion partners. Suggested fusion proteins comprise four albumin-binding moieties (albumin binding domain - ABD) comprising ABD1, e.g. 4 x ABD1 in the B domain or 4 x ABD1 at the C-terminus of the FVIII light chain.

**[0013]** WO 2012/004384 A2 discloses an albumin binding sequence. It describes a fusion protein or conjugate with the albumin binding polypeptide, wherein the second moiety may be Factor VIII.

**[0014]** WO 2013/143890 A1 discloses a compound for oral administration comprising a moiety with desired therapeutic activity and a moiety that binds to albumin.

**[0015]** WO 2014/048977 A1 discloses a class of engineered polypeptides having a binding affinity for albumin. WO 2014/064237 A1 provides albumin binding domain binding polypeptides comprising an ABD binding motif. WO 2015/091957 A1 relates to a class of engineered polypeptides having a binding affinity for albumin, wherein the polypeptides have a high resistance to proteolytic cleavage.

**[0016]** In light of the state of the art, the inventors of the present invention addressed the problem of providing recombinant FVIII proteins with increased half-life, which preferably allow a more convenient substitution therapy for the patient, allowing administration intervals of more than three days or even more than one week or more.

**[0017]** This problem is solved by the present invention, e.g., by the claimed subject-matter.

#### Description of the invention

**[0018]** The inventors have constructed new FVIII variants having a longer in vivo half-life and excellent specific activity as evidenced by different biological activity assays. These proteins further have a high level of expression and a low profile of fragments and side products. Further advantages and preferred embodiments are explained elsewhere in this description. The inventors have found that a particular arrangement of albumin binding domains contributes to an increase in in vivo half-life.

**[0019]** The invention thus provides a recombinant Factor VIII protein comprising a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion. If the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion.

**[0020]** Factor VIII in complex with von Willebrand factor (vWF) has an in vivo half-life of about 12 hours. FVIII not associated with vWF is typically degraded much more rapidly.

**[0021]** Albumin has an in vivo half-life of about 19 days. By introducing at least two albumin binding domains in the FVIII sequence, it was possible to obtain a significant half-life prolongation. Different positions and different numbers of the albumin binding moiety have been tested in order to identify the optimal positions and numbers of integrated albumin binding moieties.

**[0022]** Without intending to be bound by the theory, it is believed that albumin binding to the FVIII protein of the invention through the albumin binding domains in the specific positions described herein is particularly effective in inhibiting breakdown of the FVIII protein of the invention. This appears to increase in vivo half-life more than association with vWF associated with native FVIII in blood.

#### FVIII

**[0023]** The skilled person understands the term FVIII (or Factor VIII) and is aware of the structure and biological functions of wild type FVIII and typical variants thereof. Apart from the features specified herein, the FVIII protein of the invention may be designed as deemed appropriate and advantageous by the skilled person.

**[0024]** In particular, the Factor VIII protein of the invention should typically comprise all necessary portions and domains known to be important for biological function. For example, preferably, the FVIII protein further comprises domains corresponding to, substantially corresponding to, and/or functionally corresponding to the A and C domains of wild type FVIII, especially to A1, A2, A3, C1 and C2 domains. It may further comprise additional portions and domains. For example, preferably, the FVIII protein further comprises an a1 domain between the A1 and the A2 domains and an a2 domain C-terminal to the A2 domain. For a double chain protein, on a separate chain, or for a single chain protein, C-terminal to said domain, and, optionally, C-terminal to the B-domain or a truncated B-domain and to at least one albumin binding domain, the FVIII protein comprises at least a truncated a3 domain. Before processing, the Factor VIII protein of the invention may also comprise a signal sequence. Thus, the heavy chain portion preferably comprises the domains A1 and A2, and typically comprises the domains A1-a1-A2-a2 or A1-a1-A2-a2-B. Preferably, the B-domain of the Factor VIII protein is at least partly deleted. The light chain portion preferably comprises the domains A3 and C1 and C2, and typically comprises the domains a3-A3-C1-C2. Any or all of said domains may be wildtype (wt) FVIII domains, or they may differ from the wildtype domains, e.g., as known in the state of the art or deemed appropriate by the skilled person.

**[0025]** The domains are preferably contained in the protein in that order, i.e., from N-terminus to C-terminus of the protein.

**[0026]** While parts of the FVIII protein of the invention can be designed as desired by the skilled person, the FVIII preferably maintains a high FVIII biological activity. As shown in the examples, the invention allows generation of a FVIII

protein with a high biological activity, as measured e.g. by the chromogenic activity. Therefore, preferably the FVIII protein according to the invention has a chromogenic activity which is at least comparable to the activity of the wt FVIII protein, i.e., it has at least 50% of the specific chromogenic activity of the wt protein (SEQ ID NO: 1). Preferably, the FVIII protein according to the invention has at least 80%, at least 100 % or more than 100% of the specific chromogenic activity of the wt protein. Preferably, the chromogenic activity also is at least 80%, at least 90%, at least 100% or more than 100% of the chromogenic activity of ReFacto AF® (international non-proprietary name: Moroctocog Alfa), a commercially available B-domain deleted FVIII (Pfizer).

**[0027]** A FVIII protein according to the present invention shall have at least one biological activity or function of a wt FVIII protein, in particular with regard to the function in coagulation. The FVIII protein should be cleavable by thrombin, leading to activation. Preferably, the FVIII protein according to the invention comprises at least one thrombin recognition and/or thrombin cleavage site, wherein said thrombin recognition and/or thrombin cleavage sites may correspond to or substantially correspond to those of wild type FVIII. It is then capable of forming a complex with the activated coagulation Factor IXa and coagulation Factor X, and the light chain is capable of binding to a phospholipid bilayer, e.g., the cell membrane of (activated) platelets.

**[0028]** The biological activity of FVIII can be determined by analyzing the chromogenic, the clotting or the coagulant activity of the protein, as described herein. Typically, the chromogenic activity is taken as a measure of biological activity.

**[0029]** An increased in vivo half-life may be achieved for Factor VIII proteins of the invention that are double chain proteins. Double chain proteins which may form a basis for the FVIII proteins of the invention are known in the art, e.g., wt FVIII or B-domain deleted or truncated versions thereof, e.g., ReFacto AF®.

**[0030]** Moreover, the inventors have found an excellent increase in in vivo half-life with Factor VIII proteins of the invention that are a single chain proteins. Single chain Factor VIII proteins which may form a basis for the FVIII proteins of the invention are known in the art. In general, single chain FVIII proteins do not comprise a functional furin cleavage site and thus, before activation, remain in the circulation as a single chain. Such proteins are also disclosed in EP application No. 19173440 or taught herein.

**[0031]** As a single chain backbone for the inventive protein, a single chain FVIII molecule has been developed, in which several amino acids including the furin cleavage site (positions R1664 - R1667, wherein the signal peptide is also counted) have been deleted. The B domain is deleted to a large extent, wherein an internal fragment (at least NPP) of the B-domain is maintained and an intact Thrombin cleavage site is preceding the internal fragment. This single chain Factor VIII protein (FVIII-sc) has been shown to be more stable than wt Factor VIII. Thus, in a preferred embodiment, the recombinant Factor VIII protein of the invention comprises, in a single chain, a heavy chain portion comprising an A1 and an A2 domain and a light chain portion comprising an A3, C1 and C2 domain of Factor VIII, wherein

a) in said recombinant Factor VIII protein, 894 amino acids corresponding to consecutive amino acids between F761 and P1659 of wild type Factor VIII as defined in SEQ ID NO: 1 are deleted, leading to a first deletion;

b) said recombinant Factor VIII protein comprises, spanning the site of the first deletion, a processing sequence comprising SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site;

c) in said recombinant Factor VIII protein, at least the amino acids corresponding to amino acids R1664 to R1667 of wild type Factor VIII are deleted, leading to a second deletion; and

d) said recombinant Factor VIII protein comprises, C-terminal to the second deletion and N-terminal of the A3 domain, a second thrombin cleavage site.

**[0032]** As defined in a), in the FVIII of the invention, 894 amino acids corresponding to consecutive amino acids between F761 and P1659 of wild type Factor VIII as defined in SEQ ID NO: 1 are deleted in the Factor VIII protein of the invention, leading to a first deletion. In certain embodiments, in particular, starting from a numbering of amino acids in FVIII without deletions or insertions, the term "corresponding to" should be understood to mean "identical to".

**[0033]** For specific amino acids which may be mutated compared to the wt, an amino acid corresponding to the wild type aa is determined by an alignment e.g. using EMBOSS Needle (based on the Needleman-Wunsch algorithm; settings: MATRIX: "BLOSUM62", GAP OPEN: "20", GAP EXTEND:"0.5", END GAP PENALTY: "false", END GAP OPEN: "10", END GAP EXTEND: "0.5").

**[0034]** In order to assess sequence identities of two polypeptides, such an alignment may be performed in a two-step approach: I. A global protein alignment is performed using EMBOSS Needle (settings: MATRIX: "BLOSUM62", GAP OPEN: "20", GAP EXTEND:"0.5", END GAP PENALTY: "false", END GAP OPEN: "10", END GAP EXTEND: "0.5") to identify a particular region having the highest similarity. II. The exact sequence identity is defined by a second alignment using EMBOSS Needle (settings: MATRIX: "BLOSUM62", GAP OPEN: "20", GAP EXTEND:"0.5", END GAP PENALTY:

"false", END GAP OPEN: "10", END GAP EXTEND: "0.5") comparing the fully overlapping polypeptide sequences identified in (I) while excluding non-paired amino acids.

**[0035]** "between" excludes the recited amino acids, e.g., it means that the recited amino acids are maintained. "deletion" or "deleted" does not necessitate that the protein was actually prepared by deleting amino acids previously present in a predecessor molecule, but it merely defines that the amino acids are absent, independent from the preparation of the molecule. For example, the protein can be produced based on nucleic acids prepared by *de novo* synthesis or by genetic engineering techniques.

**[0036]** As defined in b), the

recombinant Factor VIII protein comprises, spanning the site of the first deletion, a processing sequence comprising SEQ ID NO: 2 (PRSFSQNPP) or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site. Accordingly, at least one amino acid of the processing sequence corresponds to an amino acid C-terminal to the deletion and at least one amino acid of the processing sequence corresponds to an amino acid N-terminal to the deletion. The processing sequence comprises SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, i.e., the processing sequence can be longer. In particular, the processing sequence is selected from the group comprising SEQ ID NO: 2, 4, 5, 6, 7 or 8. The inventors have found that a processing sequence of the invention enables a particularly good cleavage by thrombin.

**[0037]** In preferred embodiments, the processing sequence is no longer than SEQ ID NO: 4. The processing sequence may be directly C-terminal to sequences from the a2 domain, e.g., wt a2 domain sequences. The first N-terminal two amino acids of the processing sequence may already belong to the a2 domain. Preferably, the amino acid directly N-terminal to the processing sequence is E.

**[0038]** One amino acid in SEQ ID NO: 2 can be substituted, e.g., to reduce immunogenicity. Optionally, the F, the S C-terminal to the F, the Q or the N are substituted.

**[0039]** The processing sequence may be SEQ ID NO: 4 (PRSFSQNPPVL) or a sequence having at most one amino acid substitution in said sequence, wherein, optionally, the F, the S C-terminal to the F, the Q or the N are substituted. Moreover, the present inventors have shown that an L at the C-terminus of the processing sequence, as in SEQ ID NO: 4, 5, 6, 7 or 8, endows the FVIII with particularly good activity. One especially preferred example of a single chain FVIII protein, which may form the backbone for the protein of the invention, is shown in the examples in further detail under the name V0 (SEQ ID NO: 16). The processing sequence of the FVIII protein V0, which has been found to be particularly advantageous, consists of SEQ ID NO: 4, which is a specific embodiment of SEQ ID NO: 5-8.

**[0040]** The alternative processing sequences SEQ ID NO: 5 (PRXSQNPPVL), SEQ ID NO: 6 (PRSFXQNPPVL), SEQ ID NO: 7 (PRFSXNPPVL) and SEQ ID NO: 8 (PRSFSQXPPVL) are variants in which X can be any naturally occurring amino acid. Optionally, X is a conservative substitution compared to the corresponding amino acid in SEQ ID NO: 4, i.e. a hydrophobic amino acid is substituted by a hydrophobic amino acid, a hydrophilic amino acid is substituted by a hydrophilic amino acid, an aromatic amino acid by an aromatic amino acid, an acid amino acid by an acid amino acid and a basic amino acid by a basic amino acid.

**[0041]** As defined in c), in the FVIII protein of the invention, the amino acids corresponding to amino acids R1664 to R1667 of wild type Factor VIII are deleted, leading to a second deletion. These amino acid correspond to the furin cleavage recognition site of wt FVIII. Accordingly, the protein is essentially not cleaved by furin. In a composition, at least 80%, optionally, at least 90% or at least 95% of the FVIII protein of the invention are present in a single chain form.

**[0042]** As defined in d), the recombinant Factor VIII protein of the invention comprises, C-terminal to the second deletion and N-terminal of the A3 domain, a second thrombin cleavage site. Accordingly, upon activation, the part of the FVIII protein between the thrombin cleavage site in the processing sequence and the second thrombin cleavage site are excised from the activated FVIII protein.

**[0043]** Further, the invention provides a recombinant Factor VIII protein comprising, in a single chain, a heavy chain portion comprising an A1 and an A2 domain and a light chain portion comprising an A3, C1 and C2 domain of Factor VIII, wherein,

a) said recombinant Factor VIII protein comprises a processing sequence comprising SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site;

b) directly C-terminal to said processing sequence, said Factor VIII protein comprises a heterologous sequence comprising at least one, preferably, two albumin binding domain(s);

c) directly C-terminal to said heterologous sequence, said Factor VIII protein comprises a merging sequence having at least 90% sequence identity to SEQ ID NO: 9 (e.g., SEQ ID NO: 9); and

d) said recombinant Factor VIII protein comprises, C-terminal to SEQ ID NO: 9, a second thrombin cleavage site; and

e) said recombinant Factor VIII protein comprises, C-terminal to the light chain portion, at least one, preferably, two albumin binding domain(s).

**[0044]** Said recombinant FVIII protein may be a FVIII protein as described above. The FVIII protein typically comprises at least one further thrombin cleavage site.

**[0045]** In one embodiment, a FVIII protein of the invention that optionally is a single chain protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa1667 of SEQ ID NO: 1, and a light chain portion having at least 90% sequence identity to aa1668-aa2351 of SEQ ID NO: 1. Optionally, the respective sequence identity aa20-aa1667 of SEQ ID NO: 1 and sequence identity to aa1668-aa2351 of SEQ ID NO: 1 are at least 95%. The respective sequence identity to aa20-aa1667 of SEQ ID NO: 1 and sequence identity to aa1668-aa2351 of SEQ ID NO: 1 may be at least 98%. Optionally, the respective sequence identity to said sequences is at least 99%. The invention also provides a FVIII protein of the invention comprising a heavy chain portion having aa20-aa1667 of SEQ ID NO: 1 and a light chain portion having aa1668-aa2351 of SEQ ID NO: 1.

**[0046]** Several experiments performed by the inventors have been carried out with a single chain FVIII of the invention on the basis of the V0 single chain construct (SEQ ID NO: 16) with at least one albumin binding domain introduced C-terminal to the heavy chain portion and C-terminal to the light chain portion, as described herein. Such proteins have shown advantageous characteristics with regard to expression, stability, in vivo half-life and purification. Accordingly, a preferred FVIII protein of the invention, which may be a single chain protein, comprises a heavy chain portion having at least 90% sequence identity to aa20-aa768 of SEQ ID NO: 16, and a light chain portion having at least 90% sequence identity to aa769-aa1445 of SEQ ID NO: 16. Optionally, the respective sequence identity to aa20-aa768 of SEQ ID NO: 16 and sequence identity to aa769-aa1445 of SEQ ID NO: 16 are at least 95%. The respective sequence identity to aa20-aa768 of SEQ ID NO: 16 and sequence identity to aa769-aa1445 of SEQ ID NO: 16 may be at least 98%. Optionally, the respective sequence identity to said sequences is at least 99%. The invention also provides a FVIII protein of the invention comprising a heavy chain portion having aa20-aa768 of SEQ ID NO: 16 and a light chain portion having aa769-aa1445 of SEQ ID NO: 16.

**[0047]** wt FVIII typically is bound by vWF. vWF shields FVIII from proteolytic degradation and receptor-mediated clearance, e.g. via low-density lipoprotein (LDL) receptor-related protein (LRP1), LDL-receptor (LDLR) and heparan-sulfate proteoglycans (HSPG), within the liver (Lenting et al., 2007. J Thromb Haematol 5:1353-60). However, it has been shown that the half-life of vWF is approx. 15 h, thereby limiting the FVIII:vWF complex half-life to the vWF-related clearance pathway. The inventors found that vWF binding potency of the FVIII protein of the invention may be diminished compared to wt FVIII or ReFacto AF®, which may be explained by sterical hindrance due to albumin binding. For example, FVIII proteins of the invention may have 0%-90%, 10%-80%, 20-70%, 30-60%, or 40-50% of the binding potency of ReFacto AF® to vWF, which can be determined by an assay as described below. Preferably, in the presence of human serum albumin in physiological concentrations, said binding potency is less than 50% of the binding potency of ReFacto AF® to vWF.

**[0048]** vWF binding is mediated in particular by amino acid positions Y1683 and Y1699. To avoid vWF binding, e.g. amino acids corresponding to Y1683 and/or Y1699 of wt FVIII of SEQ ID NO: 1 may be mutated. For example, the amino acid corresponding to Y1683 and/or Y1699 of wt FVIII of SEQ ID NO: 1 may be mutated to a C or F, e.g., Y1699C or Y1699F. In particular, a mutation of the amino acid corresponding to Y1699 to F and a mutation of the amino acid corresponding to Y1683 to F, both mutations together also designated "b mutation" have been confirmed to further decrease binding of vWF to FVIII proteins of the invention. Beside the "b mutation", the inventors have additionally tested an "a mutation" comprising the amino acid substitutions Y737F, Y738F, and Y742F of wt FVIII of SEQ ID NO: 1 and a "c mutation" comprising the amino acid substitutions I2117S and R2169H of wt FVIII of SEQ ID NO: 1. Additionally, the inventors have tested combinations of "a mutation" and "b mutation" and further combinations of "a mutation" and "b mutation" and "c mutation". It was observed, that the "c mutation" negatively influenced the protein expression and functionality. The "b mutation" either alone or in combination with the "a mutation" did not influence the protein expression and functionality, but strongly decreased the binding to vWF. In comparison, the "a mutation" did not decrease the binding to vWF.

**[0049]** Thus, to further decrease vWF binding, the recombinant Factor VIII protein of the invention may have a suitable mutation as described herein, e.g., a "b mutation", i.e., a mutation of the amino acid corresponding to Y1699 to F at position 1699 and a mutation of the amino acid corresponding to Y1683 to F at position 1683 in wt Factor VIII protein of SEQ ID NO: 1. For example, the FVIII protein of the invention may comprise a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least two albumin binding domains (e.g., two) are C-terminal to the heavy chain portion and at least two albumin binding domains (e.g., two) are C-terminal to the light chain portion, wherein the FVIII protein further comprises a b mutation. Such a FVIII protein may further comprise linkers, e.g., a thrombin cleavable linker optionally flanked by a glycine-serine linker, between the albumin binding domains and other parts of the protein and between the albumin binding domains. Alternatively, such a FVIII protein does not comprise linkers. In the context of the invention, "flanked" means that the relevant portions are in a close vicinity,

preferably, with a distance of at most 10, 5 or 2 amino acids positions. Optionally, the relevant portions are immediately adjacent.

#### Albumin binding domains

**[0050]** The recombinant Factor VIII protein of the invention comprises a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion.

**[0051]** Different albumin-binding domains (ABDs) may be employed in the context of the invention. ABDs comprise or are protein domains capable of binding albumin, preferably with high affinity, e.g., with at least 50% or at least 80% of the affinity of the ABD2 to albumin. Historically, the ABD typically is a small, three-helical protein domain derived from one of various surface proteins expressed by gram-positive bacteria. For example, domains derived from streptococcal protein G and protein PAB from *Finnegoldia magna*, which share a common origin and therefore represent an interesting evolutionary system, have been thoroughly studied structurally and functionally. Their albumin-binding sites have been mapped and these domains form the basis for a wide range of protein engineering approaches. By substitution-mutagenesis they have been engineered to achieve a broader specificity, an increased stability or an improved binding affinity, respectively.

**[0052]** For example, albumin binding domains disclosed by Nilvebrant et al. (2013, Comput Struct Biotechnol J. 6: e201303009), Johansson et al. (2001, JBC 277: 8114-8120), Jacobs et al. (2015, Protein Engineering, Design and Selection 28 (10); 385-393), WO 91/01743 A1, WO 2009/016043 A2, WO 2010/054699 A1, WO 2012/004384 A2, WO 2014/048977 A1 or WO 2015/091957 A1 may be used.

**[0053]** Preferably, the albumin binding domain comprises a sequence according to SEQ ID NO: 44: LAX<sub>3</sub>AKX<sub>6</sub>X<sub>7</sub>ANX<sub>10</sub>ELDX<sub>14</sub>YGVSDFYKRLIX<sub>26</sub>KAKTVEGV EALKX<sub>39</sub>X<sub>40</sub>ILX<sub>43</sub>X<sub>44</sub>LP wherein independently of each other

X<sub>3</sub> is selected from E, S, Q and C, preferably, E;

X<sub>6</sub> is selected from E, S, C and V, preferably, E;

X<sub>7</sub> is selected from A, S and L, preferably, A;

X<sub>10</sub> is selected from A, S and R, preferably, A;

X<sub>14</sub> is selected from A, S, C and K, preferably, S;

X<sub>26</sub> is selected from D, E and N, preferably, D;

X<sub>39</sub> is selected from D, E and L, preferably, D;

X<sub>40</sub> is selected from A, E and H, preferably, A;

X<sub>43</sub> is selected from A and K, preferably, A;

X<sub>44</sub> is selected from A, S and E, preferably, A;

L in position 45 is present or absent, preferably, present; and

P in position 46 is present or absent, preferably, present.

**[0054]** Alternatively, the albumin binding domain may comprise an amino acid sequence which has at least 95 % identity to the sequence of SEQ ID NO: 44.

**[0055]** The inventors have achieved good results using an albumin binding domain designated ABD1 (SEQ ID NO: 45). It is preferred to use the sequence of ABD2 (SEQ ID NO: 46) that has been deimmunized for the human immune system, i.e., adapted to avoid immune responses in humans. If not otherwise mentioned, said albumin binding domain is used in the experiments shown herein. ABD2 may be encoded by a nucleic acid of SEQ ID NO: 57, which is codon-optimized for expression in human cells.

**[0056]** While it is possible to use different albumin binding domains at different locations in the FVIII protein, typically, all albumin binding domains in the FVIII protein will have the same sequence, preferably ABD2. Alternatively, it is possible to use different albumin binding domains at different locations within the FVIII protein in order to obtain multivalent albumin binding.

**[0057]** For example, in the FVIII protein of the invention, one albumin binding domain may be C-terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion. Alternatively, in one of the two selected positions, there may be one albumin binding domain, and two, three, four or more albumin binding domains in the other. For example, one albumin binding domain may be C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion, or one albumin binding domain may be C-terminal to the heavy chain portion and three albumin binding domains C-terminal to the light chain portion, or one albumin binding domain may be C-terminal to the heavy chain portion and four albumin binding domains C-terminal to the light chain portion.

**[0058]** In a FVIII protein of the invention, two albumin binding domains may be C-terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion, or three albumin binding domains may be C-

terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion, or four albumin binding domains may be C-terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion.

**[0059]** Preferably, the number of albumin binding domains in each of the two positions is the same. It has also been shown to be advantageous if the FVIII protein of the invention comprises at least four albumin binding domains. The inventors have found a still better increase in the half-life for Factor VIII protein of the invention comprising at least two albumin binding domains C-terminal to the heavy chain portion and at least two albumin binding domains C-terminal to the light chain portion, preferably, two albumin binding domains C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion.

**[0060]** The invention also provides Factor VIII proteins of the invention with two albumin binding domains C-terminal to the heavy chain portion and three albumin binding domains C-terminal to the light chain portion, or with two albumin binding domains C-terminal to the heavy chain portion and four albumin binding domains C-terminal to the light chain portion, or with three albumin binding domains C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion, or with four albumin binding domains C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion. Optionally, there is an even number of albumin-binding domains both C-terminal to the heavy chain and C-terminal to the light chain.

#### Linker

**[0061]** While the inventors have shown that linkers are not principally required for activity and stability of the FVIII proteins of the invention, to increase accessibility of all domains of the FVIII of the invention, in particular, access to albumin in blood, linkers were introduced into some FVIII proteins of the invention. The inventors have shown that the linkers, in particular, inclusion of at least glycine-serine linker sections, further improve expression and function. In particular, in addition to access to albumin, access for thrombin also appears to be improved. Accordingly, preferably, albumin binding domains may be separated from the heavy chain portion and/or the light chain portion and/or other albumin-binding domains by a linker, wherein, optionally, albumin-binding domains are separated from the heavy chain portion and the light chain portion and (if directly adjacent otherwise) other albumin-binding domains by a linker. It is also possible that albumin-binding domains are separated from the heavy chain portion and the light chain portion and (if directly adjacent otherwise) other albumin-binding domains by a linker, except that there is no linker N-terminal to the light chain, because the  $\alpha 3$  domain contains a thrombin cleavage site.

**[0062]** In one preferred embodiment, in the recombinant Factor VIII protein of the invention, the linker comprises a thrombin-cleavable linker section. Optionally, a thrombin cleavable linker has the sequence of SEQ ID NO: 39 (abbr. L). Further thrombin cleavable sites are known in the art, e.g., disclosed in Gallwitz et al. (2012, PLoS ONE 7 (2): e31756). Thrombin cleavable linkers may thus also comprise any of these cleavable sites. Thrombin-cleavable linkers have the advantage that in generation of the active protein, i.e., after activation by thrombin, the linkers may be cleaved and, consequently, the albumin binding domains may be removed from the active protein.

**[0063]** Alternatively, in the recombinant Factor VIII protein of the invention, uncleavable glycine-serine linker sections may be used to introduce flexible, sterical distance between motifs to avoid structural influences. Thus, optionally, the linker comprises a glycine-serine linker section that optionally has the sequence of SEQ ID NO: 40 (abbr. G1, preferred) or SEQ ID NO: 41 (abbr. G2). The linker G1 may, e.g., be encoded by SEQ ID NO: 58. The linker G2 may, e.g., be encoded by SEQ ID NO: 59.

**[0064]** In preferred embodiments, different linker sections are combined. E.g. uncleavable linker sections were used to flank a central thrombin cleavable linker section to maintain thrombin accessibility of the thrombin cleavable linker section. Thus, in some embodiments, the linker comprises a thrombin cleavable linker section flanked on each side by a glycine-serine linker section, wherein said combined linker optionally has the sequence of SEQ ID NO: 42 or SEQ ID NO: 43, preferably, SEQ ID NO: 42.

**[0065]** The polynucleotide sequence for all linkers preferably is codon-optimized for human use. Exemplary codon-optimized sequences are provided herein in the context of FVIII proteins of the invention.

#### Specific FVIII proteins of the invention

**[0066]** All FVIII proteins of the invention demonstrated good in vitro functionality, wherein the FVIII proteins showed reduced vWF binding in correlation to increasing numbers of albumin binding domains. vWF has a major impact on the half-life of FVIII. It was found that shielding FVIII from vWF by albumin positively influences the half-life of the FVIII protein. A broad distribution of albumin binding domains with one position between heavy chain and light chain and one position at the C-terminus of the protein was shown to enhance the shielding of FVIII from vWF.

**[0067]** Preferably, the recombinant Factor VIII protein the invention comprises one albumin binding domain between the heavy chain portion and the light chain portion and one albumin binding domain C-terminal to the light chain portion,



wherein the sequence has at least 70%, optionally, at least 80%, at least 90%, at least 95%, at least 99% or 100% sequence identity to SEQ ID NO: 47. Preferably, said protein is a single chain protein. The single chain protein having SEQ ID NO: 47 is designated ADLCLD\_SC. It was shown to have an in vivo half-life increased by a factor of about 1.5 compared to ReFacto AF®.

**[0068]** In an especially preferred embodiment, the recombinant Factor VIII protein of the invention comprises at least two albumin binding domains between the heavy chain portion and the light chain portion and at least two albumin binding domain C-terminal to the light chain portion, wherein the protein has at least 80% sequence identity, optionally, at least 90%, at least 95% or at least 99% sequence identity to any of SEQ ID NO: 48, 49, 51. Preferably, the recombinant Factor VIII protein has at least 80% sequence identity, optionally, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 48. Preferably, said protein is a single chain protein.

**[0069]** The recombinant Factor VIII protein may also have at least 80% sequence identity, optionally, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 49. Preferably, said protein is a single chain protein.

**[0070]** The recombinant Factor VIII protein may also have at least 80% sequence identity, optionally, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 51. Preferably, said protein is a single chain protein.

**[0071]** For example, the invention provides a recombinant FVIII protein having SEQ ID NO: 48 (AD2CD2\_SC), SEQ ID NO: 49 (AD2CD2woL\_SC), or SEQ ID NO: 51 (AbD2CD2\_SC). Such FVIII proteins have been shown to have a particularly extended in vivo half-life, e.g., for AD2CD2\_SC, an in vivo half-life extended by a factor of 2.5 has been found in hemophilia A mice and a half-life extension of factor 4 has been found in albumin-deficient, transgenic neonatal Fc-receptor mice (see Examples). For AbD2CD2\_SC, an in vivo half-life extended by a factor of 2.2 has been found. An increase in half-life can be analyzed on the level of FVIII antigen or on the level of activity, e.g., chromogenic activity, or both. Preferably, it is analyzed on the level of chromogenic activity.

**[0072]** The invention thus provides FVIII proteins of the invention, wherein the in vivo half-life of the recombinant Factor VIII protein is prolonged (i.e. increased) by a factor of at least 1.2, preferably, by a factor of at least 1.5, optionally, at least 2 or at least 2.5 in comparison to a recombinant Factor VIII protein of SEQ ID NO: 28 (ReFacto AF®). While the increase in in vivo half-life may be analyzed in model systems, e.g., mice, rats or dogs, such as in hemophilia A mice or albumin-deficient Tg32 mice having a knock-out of murine albumin and expressing human FcRn a-chain instead of the murine one (B6.Cg-Alb<sup>em12</sup>Mvw Fcgr<sup>tm1Dcr</sup> Tg(FCGRT)32Dcr/MvwJ), the observed increase in in vivo half-life may be underestimated, because human albumin has a longer half-life than e.g. murine albumin, and it is expected that an increase seen in a murine model will be still more pronounced in humans.

**[0073]** A fusion partner may be employed to extend the in vivo plasma half-life of the FVIII protein of the invention. In one embodiment, the recombinant Factor VIII protein of the invention is a fusion protein with at least one further heterologous fusion partner in addition to the albumin binding moiety, preferably with a further fusion partner extending the in vivo plasma half-life of the FVIII protein. The fusion partner may e.g. be selected from the group comprising an Fc region, albumin, PAS polypeptides, HAP polypeptides, the C-terminal peptide of the beta subunit of chorionic gonadotropin, and combinations thereof. The FVIII protein may alternatively or additionally be covalently linked to non-protein fusion partners such as albumin-binding small molecules (e.g., dabigatran), PEG (polyethyleneglycol) and/or HES (hydroxyethyl starch). PAS polypeptides or PAS sequences are polypeptides comprising an amino acid sequence comprising mainly alanine and serine residues or comprising mainly alanine, proline and serine residues, the PAS sequences forming a random coil conformation under physiological conditions, as defined in WO 2015/023894. HAP polypeptides or sequences are homo-amino acid polymer (HAP), comprising e.g., repetitive sequences of glycine or glycine and serine, as defined in WO 2015/023894. Potential fusions, fusion partners and combinations thereof are described in more detail e.g., in WO 2015/023894.

**[0074]** Optionally, for certain therapeutic applications, the recombinant FVIII protein may be fused to an Fc region. A fusion to an Fc region may be used to extend the half-life and reduce immunogenicity.

**[0075]** Optionally, said heterologous fusion partner may be inserted directly N-terminal or directly C-terminal to one of the albumin binding domains, e.g., C-terminal to the heavy chain, and/or C-terminal to the C2-domain, or C-terminal to the albumin binding domain(s) C-terminal to the heavy chain or C-terminal to the albumin binding domain(s) C-terminal to the heavy chain. These locations have been found by the inventors to be advantageous for fusion, while maintaining good biological activity of the FVIII protein. Optionally, the fusion protein further comprises at least one linker.

**[0076]** The protein may further be glycosylated and/or sulfated. Preferably, post-translational modifications such as glycosylation and/or sulfation of the protein occur in a human cell. A particularly suitable profile of post-translational modifications can be achieved using human cell lines for production, e.g. CAP cells, in particular CAP-T cells or CAP-Go cells (WO 2001/36615; WO 2007/056994; WO 2010/094280; WO 2016/110302). CAP cells, available from Cevac Pharmaceuticals GmbH (Cologne, Germany), originate from human amniocytes as they were isolated trans-abdominally during routine amniocentesis. Obtained amniocytes were transformed with adenoviral functions (E1A, E1B, and pIX functions) and subsequently adapted to growth in suspension in serum-free medium.

**[0077]** During post-translational modification, the FVIII protein of the invention may be sulfated, e.g., on one, two, three, four, five or six tyrosines in the acidic regions a1, a2 and a3.

**[0078]** Optionally, the recombinant Factor VIII protein of the invention is a de-immunized protein, i.e., the protein has a reduced immunogenicity as compared to wt FVIII in hemophilia patients. For example, certain mutations, preferably, substitutions, have been introduced to avoid the presence of epitopes which can be presented on human HLA molecules, preferably, common human HLA molecules.

#### Nucleic acids and host cells

**[0079]** The invention also provides a nucleic acid encoding a recombinant Factor VIII protein of the invention. Said nucleic acid may be an expression vector, e.g., suitable for expression of said recombinant Factor VIII protein in a mammalian cell, such as a human cell, such as a CAP cell.

**[0080]** The nucleic acid preferably encodes the FVIII with an N-terminal signal sequence, e.g., the 19 aa signal sequence of SEQ ID NO: 1. Preferred nucleic acids of the invention encode a recombinant FVIII protein having SEQ ID NO: 47 (ADLCLD\_SC), SEQ ID NO: 48 (AD2CD2\_SC), SEQ ID NO: 49 (AD2CD2woL\_SC), SEQ ID NO: 50 (AD2CD2woLG\_SC) or SEQ ID NO: 51 (AbD2CD2\_SC). They may be SEQ ID NO: 52-56. The nucleic acids of the invention may be DNA molecules or RNA molecules. The nucleic acids may be optimized for expression in the respective host cell, e.g., in a human cell, e.g., a CAP cell.

**[0081]** The expression vector comprises the sequence encoding the FVIII protein, preferably, in codon-optimized form, under the functional control of a suitable promoter, which may be a constitutive or an inducible promoter. The promoter may be a promoter not associated with expression of FVIII in nature, e.g., EF-1 alpha or a heterologous promoter, e.g., CMV or SV40. It may further comprise pro- and/or eukaryotic selection markers, such as ampicillin resistance and dihydrofolate reductase (dhfr), and origins of replication, e.g., an SV40 origin and/or a pBR322 origin. "codon-optimized" means optimized for expression in the host cell, preferably, for expression in a human host cell.

**[0082]** In certain embodiments, the nucleic acid may be a vector suitable for gene therapy, e.g., for gene therapy of a human patient. Vectors suitable for gene therapy are known in the art, e.g., virus-based vectors e.g., based on adenovirus or adeno-associated virus (AAV) or based on retrovirus, such as lentiviral vectors etc., or non virus-based vectors such as but not limited to small plasmids and minicircles or transposon-based vectors. An AAV-based vector of the invention may e.g., be packaged in AAV particles for gene therapy of Hemophilia A patients.

**[0083]** The invention also provides a host cell comprising a nucleic acid of the invention. The host cell may be a bacterial cell, a plant cell, a fungal cell, a yeast cell or an animal cell. Preferably, the host cell is an animal cell, in particular, a mammalian cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said cell. The host cell preferably is a human cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said human cell. The cell may be transiently or stably transfected with the nucleic acid of the invention. The cell may be a cell line, a primary cell or a stem cell. For production of the protein, the cell typically is a cell line such as a HEK cell, such as a HEK-293 cell, a CHO cell, a BHK cell, a human embryonic retinal cell such as Crucell's Per.C6 or a human amniocyte cell such as CAP. For treatment of human patients with the protein, the host cell preferably is a human cell, e.g., a HEK293 cell line or a CAP cell line (e.g. a CAP-T cell or a CAP-Go cell). The inventors have found that in a CAP cell line, a particularly high single chain content of FVIII protein of the invention is produced. Among the CAP cells, CAP-T cells are preferred for transient expression, while CAP-Go cells may be used for creation of stable cell lines conveying an advantageous glycosylation profile to the FVIII molecule.

**[0084]** The cell may be an autologous cell of a Hemophilia A patient, in particular, a human Hemophilia A patient, suitable for producing FVIII in the patient after transfection and reintroduction into the patient's body. The cell may be a stem cell, e.g., a hematopoietic stem cell, but preferably it is not an embryonic stem cell, in particular when the patient is a human. The cell may also be hepatocyte, a liver sinusoidal endothelial cell or a thrombocyte.

**[0085]** Cell lines expressing the protein of the invention may also be used in a method of preparing the protein of the invention, comprising cultivating said cells under conditions suitable for expression of the FVIII protein and purifying said protein, e.g., using a plurality of methods known to the skilled person, such as described herein. Such purification methods may comprise standard harvesting procedures for cell removal, e.g. centrifugation, followed by chromatography steps, e.g. affinity chromatography, and methods for exchanging the FVIII proteins into a suitable buffer. The invention thus also provides a method for preparing a Factor VIII protein, comprising culturing the host cell of the invention under conditions suitable for expression of the Factor VIII protein and isolating the Factor VIII protein, wherein the method optionally comprises formulating the Factor VIII protein as a pharmaceutical composition.

#### Pharmaceutical compositions

**[0086]** The invention provides a pharmaceutical composition comprising the recombinant Factor VIII protein of the invention, the nucleic acid of the invention or the host cell of the invention. Such pharmaceutical compositions may comprise suitable excipients or carriers, e.g., a buffer, a stabilizing agent, a bulking agent, a preservative, another (e.g., recombinant) protein or combinations thereof. In the context of the invention, if not explicitly stated otherwise, "a" is

understood to mean one or more.

**[0087]** A suitable buffer for formulation of the protein of the invention may e.g. contain 205 mM NaCl, 5.3 mM CaCl<sub>2</sub>, 6.7 mM L-Histidine, 1.3 % Sucrose and 0.013 % Tween 20 in distilled water and have a pH of 7.0 (FVIII formulation buffer). Said buffer is used in the experiments described herein if not otherwise stated. Formulations of FVIII may be sterile, e.g., sterile filtered, in particular for in vivo use.

**[0088]** Optionally, the pharmaceutical composition of the invention comprising FVIII protein further comprises albumin, preferably, for a human patient, human serum albumin. Albumin may, e.g., be in a concentration of 0.1-10% w/w, such as 1-5% human serum albumin (w/w). Albumin may bind to the ABD of the FVIII proteins of the invention either before administration, or after administration to a human subject.

**[0089]** The pharmaceutical composition may be formulated as desired appropriate by the skilled person, e.g., for intravenous (i.v.) or subcutaneous application, intraperitoneal or intramuscular application. Generally, it is for administration as slow i.v. push bolus injection. Continuous infusion is indicated e.g., for patients requiring admission for severe bleeds or surgical procedures. Oral application, which may contribute to tolerance induction, is also possible, e.g., after expression in plants. The pharmaceutical composition may be for slow release.

**[0090]** Pharmaceutical compositions comprising FVIII can be lyophilized.

**[0091]** Due to the increase in half-life in vivo, the pharmaceutical compositions of the invention may be administered at longer intervals than previous FVIII compositions. For example, they may be for use in administration every 5 to 14 days, preferably, every 7 to 10 days.

**[0092]** Dosages and treatment schemes may be chosen as appropriate, e.g., for prophylaxis of bleeding or with intermittent, on-demand therapy for bleeding events. Decisions on dosing may be made by the physician. Dosing depends on the patent, e.g., weight, FVIII status, severity of disease etc. For example, the FVIII of the invention may be administered in dosages of 0.5 to 250 IU/kg body weight every 0.5 to 14 or every 6-7 days intravenously depending on the severity of the disease, typically, 0.5 to 200 IU/kg body weight.

**[0093]** The invention also provides a pharmaceutical composition comprising the FVIII protein of the invention in combination with an immunosuppressive agent (e.g., methylprednisolone, prednisolone, dexamethasone, cyclophosphamide, rituximab, and/or cyclosporin), and/or it may be for administration at substantially the same time (e.g. within five minutes to within 12 hours) with such an agent. The invention thus also provides a kit comprising, in addition to a FVIII protein of the invention, optionally, combined with albumin, an immunosuppressive agent, e.g., an immunosuppressive agent selected from the group comprising methylprednisolone, prednisolone, dexamethason, cyclophosphamide, rituximab, and/or cyclosporin.

**[0094]** The pharmaceutical composition, e.g., comprising the protein of the invention, may be for use in treating a patient in need thereof, in particular, a Hemophilia A patient, e.g., a patient with acquired hemophilia involving an autoimmune response to FVIII or a congenital Hemophilia A patient. Mammals such as mice or dogs may be treated with the pharmaceutical composition of the invention, but the patient typically is a human patient.

**[0095]** The pharmaceutical composition of the invention may also be used for treatment of a patient previously already treated with a recombinant and/or plasmatic Factor VIII protein. In a patient who has an antibody including an inhibitory antibody response to a recombinant and/or plasmatic Factor VIII protein, the pharmaceutical compositions may, e.g., be used for immune tolerance induction (ITI) treatment. The compositions of the invention may thus also be used for rescue ITI. The pharmaceutical compositions may also be advantageously used in a patient who has had an antibody response including an inhibitory antibody response to a recombinant and/or plasmatic Factor VIII protein, e.g., who has been treated by ITI. The pharmaceutical compositions may also be advantageously used in a patient who has had an antibody response including an inhibitory antibody response to a recombinant and/or plasmatic Factor VIII protein, who has not been treated by ITI.

**[0096]** The invention also provides a vial comprising the pharmaceutical composition of the invention, e.g., a syringe. The syringe may be a pre-filled syringe, e.g., a ready-to-use syringe.

**[0097]** The inventors contemplate that, in the context of gene therapy, due to the presence of the albumin binding domains, long-term expression and folding of the FVIII protein of the invention may be improved compared to expression of conventional FVIII.

**[0098]** All publications cited herein are fully incorporated herewith. The invention is further illustrated by the following embodiments, figures and examples, which are not to be understood as limiting the scope of the invention.

#### Embodiments

**[0099]** In summary, in a first embodiment, the invention provides a recombinant Factor VIII protein comprising a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion, wherein, if the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion.

**[0100]** In a second embodiment, the recombinant Factor VIII protein of the first embodiment (embodiment 1) is a single chain protein.

**[0101]** In a third embodiment, the recombinant Factor VIII protein of embodiment 1 is a double chain protein.

**[0102]** In a fourth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion.

**[0103]** In a fifth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion.

In a sixth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and three albumin binding domains are C-terminal to the light chain portion. In a seventh embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and four albumin binding domains are C-terminal to the light chain portion.

**[0104]** In an eighth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, two albumin binding domains are C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion. In a ninth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, three albumin binding domains are C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion. In a tenth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, four albumin binding domains are C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion.

**[0105]** In an eleventh embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, at least two albumin binding domains are C-terminal to the heavy chain portion and at least two albumin binding domains are C-terminal to the light chain portion, preferably, two albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion. In a twelfth embodiment, in the recombinant Factor VIII protein of embodiment 11, two albumin binding domains are C-terminal to the heavy chain portion and three albumin binding domains are C-terminal to the light chain portion. In a thirteenth embodiment, in the recombinant Factor VIII protein of embodiment 11, two albumin binding domains are C-terminal to the heavy chain portion and four albumin binding domains are C-terminal to the light chain portion. In a fourteenth embodiment, in the recombinant Factor VIII protein of embodiment 11, three albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion. In a fifteenth embodiment, in the recombinant Factor VIII protein of embodiment 11, four albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion.

**[0106]** In a sixteenth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-15, albumin-binding domains are separated from the heavy chain portion and/or the light chain portion and/or other albumin-binding domains by a linker, wherein, preferably, albumin-binding domains are separated from the heavy chain portion and the light chain portion and other albumin-binding domains by a linker.

**[0107]** In a seventeenth embodiment, in the recombinant Factor VIII protein of embodiment 16, the linker comprises a Thrombin-cleavable linker section that optionally has the sequence of SEQ ID NO: 39.

**[0108]** In an eighteenth embodiment, in the recombinant Factor VIII protein of any of embodiments 16 or 17, the linker comprises a glycine-serine linker section that optionally has the sequence of SEQ ID NO: 40 or SEQ ID NO: 41.

**[0109]** In a nineteenth embodiment, in the recombinant Factor VIII protein of any of embodiments 16-18, said linker is a combination of different linker sections, e.g. the linker comprises a Thrombin-cleavable linker section flanked on each side by a glycine-serine linker section, wherein said linker optionally has the sequence of SEQ ID NO: 42 or SEQ ID NO: 43.

**[0110]** In a twentieth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-19, the albumin binding domain comprises a sequence according to SEQ ID NO: 44.

**[0111]** In a twenty-first embodiment, in the recombinant Factor VIII protein of embodiment 20, the albumin binding domain comprises a sequence according to SEQ ID NO: 46.

**[0112]** In a twenty-second embodiment, in the recombinant Factor VIII protein of any of embodiments 1-21, the heavy chain portion comprises the domains A1 and A2, and optionally comprises the domains A1-a1-A2-a2 or A1-a1-A2-a2-B.

**[0113]** In a twenty-third embodiment, in the recombinant Factor VIII protein of any of embodiments 1-22, the light chain portion comprises the domains A3 and C1 and C2, and optionally comprises the domains a3-A3-C1-C2.

**[0114]** In a twenty-fourth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-23, the B-domain of the Factor VIII protein is at least partly deleted.

**[0115]** In a twenty-fifth embodiment, the recombinant Factor VIII protein of any of embodiments 1-24 comprises, in a single chain, a heavy chain portion comprising an A1 and an A2 domain and a light chain portion comprising an A3, C1 and C2 domain of Factor VIII, wherein

a) in said recombinant Factor VIII protein, 894 amino acids corresponding to consecutive amino acids between F761 and P1659 of wild type Factor VIII as defined in SEQ ID NO: 1 are deleted, leading to a first deletion;

b) said recombinant Factor VIII protein comprises, spanning the site of the first deletion, a processing sequence comprising SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site;

c) in said recombinant Factor VIII protein, at least the amino acids corresponding to amino acids R1664 to R1667 of wild type Factor VIII are deleted, leading to a second deletion; and

d) said recombinant Factor VIII protein comprises, C-terminal to the second deletion and N-terminal of the A3 domain, a second thrombin cleavage site.

**[0116]** In a twenty-sixth embodiment, the recombinant Factor VIII protein of any of embodiments 1-25 that optionally is a single chain protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa768 of SEQ ID NO: 16 and a light chain portion having at least 90% sequence identity to aa769-aa1445 of SEQ ID NO: 16, wherein said sequence identities preferably are at least 95%, at least 98% or 100%.

**[0117]** In a twenty-seventh embodiment, the recombinant Factor VIII protein of any of embodiments 1-26 that optionally is a single chain protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa1667 of SEQ ID NO: 1 and a light chain portion having at least 90% sequence identity to aa1668-aa2351 of SEQ ID NO: 1, wherein said sequence identities optionally are at least 95%, at least 98% or 100%. In a twenty-eighth embodiment, the recombinant Factor VIII protein of any of embodiments 1-4 and 16-27, comprises one albumin binding domain between the heavy chain portion and the light chain portion and one albumin binding domain C-terminal to the light chain portion, wherein the sequence has at least 70% sequence identity to SEQ ID NO: 47.

**[0118]** In a twenty-ninth embodiment, the recombinant Factor VIII protein of any of embodiments 11-28 is a single chain protein comprising at least two albumin binding domains between the heavy chain portion and the light chain portion and at least two albumin binding domain C-terminal to the light chain portion, wherein the protein has at least 80% sequence identity, optionally, at least 90% sequence identity, at least 95% sequence identity or at least 98% sequence identity to any of SEQ ID NO: 48, 49 or 51. In a thirtieth embodiment, the recombinant Factor VIII protein of embodiment 29 has at least 80% sequence identity to SEQ ID NO: 48, e.g., it has SEQ ID NO: 48 or SEQ ID NO: 51.

**[0119]** In a thirty-first embodiment, the recombinant Factor VIII protein of any one of embodiments 1-30 has a "b mutation", i.e., a mutation of the amino acid corresponding to Y1699 to F at position 1699 and a mutation of the amino acid corresponding to Y1683 to F at position 1683 in wt Factor VIII protein of SEQ ID NO: 1.

**[0120]** In a thirty-second embodiment, the in vivo half-life of the recombinant Factor VIII protein of any one of embodiments 1-31 in a human subject is prolonged by a factor of at least 1.2, preferably, by a factor of at least 1.5, optionally, at least 2 or at least 2.5 in comparison to a recombinant Factor VIII protein of SEQ ID NO: 28.

**[0121]** In a thirty-third embodiment, the recombinant Factor VIII protein of any one of embodiments 1-32 is a fusion protein with at least one fusion partner selected from the group consisting of an Fc region, albumin, PAS polypeptides, HAP polypeptides, the C-terminal peptide of the beta subunit of chorionic gonadotropin, polyethylenglycol, and hydroxyethyl starch.

**[0122]** In a thirty-fourth embodiment, the recombinant Factor VIII protein of any one of embodiments 1-33 is a de-immunized protein.

**[0123]** In a thirty-fifth embodiment, the invention provides a nucleic acid encoding a recombinant Factor VIII protein of any one of embodiments 1-34. In a thirty-sixth embodiment, the nucleic acid of embodiment 35 is an expression vector, preferably, suitable for expression of said recombinant Factor VIII protein in a mammalian cell, preferably, in a human cell, such as a CAP cell.

**[0124]** In a thirty-seventh embodiment, the invention provides a host cell comprising a nucleic acid of any of embodiments 35 or 36. In a thirty-eighth embodiment, the host cell of embodiment 37 is a mammalian cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said cell, preferably, a human cell selected from the group comprising a Hek293 cell or a CAP cell (e.g., CAP-T cell or CAP-Go cell).

**[0125]** In a thirty-ninth embodiment, the invention provides a method of preparing a recombinant Factor VIII protein, comprising culturing the host cell of embodiments 37 or 38 under conditions suitable for expression of the Factor VIII protein and isolating the recombinant Factor VIII protein, wherein the method optionally comprises formulating the Factor VIII protein as a pharmaceutical composition.

**[0126]** In a fortieth embodiment, the invention provides a pharmaceutical composition comprising the recombinant Factor VIII protein of any of embodiments 1-34, the nucleic acid of any of embodiments 35-36 or the host cell of any of embodiments 37-38. In a forty-first embodiment, the pharmaceutical composition of embodiment 40 further comprises a biologically acceptable carrier such as water or a buffer, optionally, at a physiologic pH, preferably, FVIII formulation buffer, and/or pharmaceutically acceptable excipients. In a forty-second embodiment, the pharmaceutical composition of any of embodiments 40 or 41 further comprises albumin, e.g., 0.1-5%, such as 1% human serum albumin.

**[0127]** In a forty-third embodiment, the invention provides a pharmaceutical composition of any of embodiments 40-42

or a kit further comprising an immunosuppressive agent, e.g., an immunosuppressive agent selected from the group comprising methylprednisolone, prednisolone, dexamethason, cyclophosphamide, rituximab, and/or cyclosporin.

**[0128]** The invention further provides, as a forty-fourth embodiment, a pharmaceutical composition of any of embodiments 40-43 for use in treatment of hemophilia A, wherein, optionally, the treatment is immune tolerance induction (ITI).

In a forty-fifth embodiment, the pharmaceutical composition of any of embodiments 40-44 is for use in treating a patient with Hemophilia A selected from the group comprising a patient not previously treated with any Factor VIII protein, a patient previously treated with a Factor VIII protein, a patient who has an antibody response including an inhibitory antibody response to a Factor VIII protein, and a patient who has had an antibody response including an inhibitory antibody response to a Factor VIII protein who has been treated by ITI, or who has not been treated by ITI.

**[0129]** In a forty-sixth embodiment, the pharmaceutical composition of any of embodiments 40-45 is for administration every 5 to 14 days, preferably, every 7 to 10 days.

**[0130]** In a forty-seventh embodiment, the invention provides a vial, e.g., a prefilled or ready-to use syringe, comprising the pharmaceutical composition of any of embodiments 40-46.

**[0131]** In a forty-eighth embodiment, the invention provides a method of treatment, comprising administering an effective amount of the pharmaceutical composition of any of embodiments 40-46 to a patient in need thereof, e.g., a patient with hemophilia A, which may be selected from the patient groups defined herein.

SEQ ID NO: 1	wt human FVIII
SEQ ID NO: 2	processing sequence in preferred single chain constructs
SEQ ID NO: 4	processing sequence in V0
SEQ ID NO: 5	variant processing sequence, X can be varied for de-immunization
SEQ ID NO: 6	variant processing sequence, X can be varied for de-immunization
SEQ ID NO: 7	variant processing sequence, X can be varied for de-immunization
SEQ ID NO: 8	variant processing sequence, X can be varied for de-immunization
SEQ ID NO: 9	merging sequence, e.g., in V0
SEQ ID NO: 16	single chain FVIII V0
SEQ ID NO: 28	6rs-REF
SEQ ID NO: 39	Thrombin-cleavable linker
SEQ ID NO: 40	glycine-serine linker G1, G in position 14 is present or absent
SEQ ID NO: 41	glycine-serine linker G2
SEQ ID NO: 42	Thrombin-cleavable linker flanked on each side by a glycine-serine linker, strictly repetitive
SEQ ID NO: 43	Thrombin-cleavable linker flanked on each side by a glycine-serine linker, non-repetitive
SEQ ID NO: 44	ABD consensus sequence, see above
SEQ ID NO: 45	ABD1
SEQ ID NO: 46	ABD2
SEQ ID NO: 47	ADLCLD_SC aa
SEQ ID NO: 48	AD2CD2_SC aa
SEQ ID NO: 49	AD2CD2woL_SC aa
SEQ ID NO: 50	AD2CD2woLG_SC aa
SEQ ID NO: 51	AbD2CD2_SC aa
SEQ ID NO: 52	ADLCLD_SC na
SEQ ID NO: 53	AD2CD2_SC na
SEQ ID NO: 54	AD2CD2woL_SC na
SEQ ID NO: 55	AD2CD2woLG_SC na
SEQ ID NO: 56	AbD2CD2_SC na
SEQ ID NO: 57	optimized DNA sequence encoding SEQ ID NO: 46
SEQ ID NO: 58	exemplary DNA encoding Glycine-serine linker G1 of SEQ ID NO: 40
SEQ ID NO: 59	exemplary DNA encoding Glycine-serine linker G2 of SEQ ID NO: 41

## Figure Legends

**[0132]**

Fig. 1 shows the human albumin binding of ADLCLD\_SC, a FVIII protein of the invention comprising two albumin-

binding domains in comparison to FVIII 6rs-Ref, a protein having the ReFacto AF sequence. Both FVIII proteins were tested in the presence and absence of HSA via the albumin binding capacity assay as described.

Fig. 2 shows the von-Willebrand factor (vWF) binding capacity of different FVIII-albumin-binding-domain fusion proteins in relation of ReFacto AF. All FVIII molecules were tested for their vWF binding in either the presence or absence of human albumin. The more albumin-binding domains were incorporated into FVIII, the lower was the binding to vWF in general. The presence of human albumin dramatically decreased the binding to vWF.

Fig. 3 Comparison of unpurified FVIII-ABD fusion variants and FVIII controls for their in vitro functionality. Cell culture supernatants of CAP-T cells expressing the double chain FVIII molecule 6rs-REF, the single chain FVIII molecule AC\_SC, and the FVIII-ABD fusion molecules AD2CD2\_SC, AD2CD2woLG\_SC, AD2CD2wL\_SC, ACD4woLG\_SC, and ACL(GD)<sub>4</sub>\_SC were analyzed for chromogenic FVIII activity (A), FVIII clotting activity induced by Actin FSL (B) and FVIII antigen levels indicating total FVIII protein amount (C). Specific chromogenic activity was calculated as chromogenic FVIII activity to FVIII antigen ratio displayed in % (D). Specific clotting activity was calculated as FVIII clotting activity to FVIII antigen ratio displayed in % (E). n=2.

Fig. 4 Western blot analysis of unpurified FVIII-ABD fusion variants and FVIII control proteins for assessing structural properties. Cell culture supernatants of CAP-T cells expressing the double chain FVIII molecule 6rs-REF, the single chain FVIII molecule AC\_SC, and the FVIII-ABD fusion molecules AD2CD2\_SC, AD2CD2woLG\_SC, AD2CD2wL\_SC, and ACD4woLG\_SC were separated by non-reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequent blotting onto a PVDF membrane was performed. A purified Sheep anti-Human Factor VIII primary antibody and CF680-conjugated donkey anti-sheep IgG (H&L) antibody was used for detection. For size determination, Precision Plus All Blue was applied as marker.

Fig. 5 demonstrates the in vivo pharmacokinetics of AD2CD2\_SC compared to ReFacto AF after a single injection of 200 U/kg FVIII (with 1% human albumin) into mice having a knock-out for murine albumin and expressing the  $\alpha$ -chain of human instead of murine neonatal Fc-receptor. Determined FVIII antigen values were normalized and are shown in percent over time.

Fig. 6 demonstrates the in vivo pharmacokinetics of AD2CD2\_SC compared to ReFacto AF after a single injection of 30 U FVIII Antigen/kg formulated with either 1 or 10% human albumin into Göttingen minipigs. Plasma samples were withdrawn and FVIII antigen levels were measured by ELISA. Mean FVIII antigen levels are shown in U/ml over time in hours. n=3 minipigs per group.

Fig. 7 shows the total bleeding time (first column of each group, left Y-axis) and the total blood loss (second column of each group, right Y-axis) after tail transection of hemophilia A mice which were administered 20 h earlier with either Vehicle Control, ReFacto AF, Elocate, AD2CD2\_SC or ADLCLD\_SC. Non-hemophilia C57BL/6NCrl mice were treated with 0.9% NaCl and used as control.

## Examples

### 1. Evaluation of Factor VIII proteins with additions of albumin-binding domains for the development of a new hemophilia A therapeutic

#### Material and methods

##### Preparation of constructs

**[0133]** Experiments were performed to find and develop a suitable backbone for integration of the albumin-binding domains. The experiments were done on the basis of a B-domain deleted version of FVIII and single chain variants of FVIII. The basic double chain construct was a codon-optimized sequence of ReFacto AF® (Pfizer), wherein for simplifying cloning, 6 restriction sites were added through silent mutations, but some of these restriction sites were again excluded due to codon-optimization. The basic double chain sequence is 6rs-REF (SEQ ID NO: 28). The basic single chain construct used was V0 (SEQ ID NO: 16, EP19173440)

**[0134]** Firstly, the ABD protein sequence (Affibody AB, Solna, Sweden) was taken as a basis for design of the DNA sequence. If not mentioned otherwise, the ABD2 sequence was used. Moreover, codon optimized linkers were developed, which are partly cleavable by thrombin. If not otherwise stated, the glycine-serine linker was G1 and the thrombin-cleavable linker was L. Table 1 below demonstrates structures of fusion proteins with albumin-binding domains (ABD)

for single chain molecules.

**[0135]** For the constructs encoding the FVIII of the invention and comparative constructs also analysed in this context, either the complete FVIII sequence or DNA regions carrying approx. 700-1200 bp from the FVIII a2 domain to the A3 domain were synthesized. The synthesized DNA was codon-optimized for the total target gene. The a2 to A3 DNA fragments were 5' terminally flanked by an EcoRV restrictions site, and 3' terminally flanked by an EcoRI restriction site, and these restriction sites were also present in the basic FVIII sequence used. For C-terminal fusion to the light chain, DNA fragments carrying approx. 1500-2100 bp were synthesized also in a codon-optimized form. Such DNA fragments were 5' terminally flanked by an EcoRI restrictions site within the A3 domain, and 3' terminally flanked by a NotI restriction site. Restriction of the DNA inserts and the FVIII backbone plasmid allowed for targeted ligation and generation of FVIII single chain plasmids. Completely synthesized FVIII DNA was 5' terminally flanked by a HindIII restrictions site, and 3' terminally flanked by a NotI restriction site.

**[0136]** By transformation of *E.coli* K12 with said plasmids, expansion of transformed bacteria under ampicillin selection and plasmid preparation, large amounts of the plasmids could be prepared. Genetic engineering work was carried out by Thermo Fisher Scientific after design with VectorNTI Software (Thermo Fisher Scientific, Massachusetts, USA).

#### Cultivation of CAP-T cells

**[0137]** For analyzing the candidates for new recombinant FVIII molecules, the constructs, integrated in expression vectors, were transiently and stably expressed in human cell lines. The preferred cell lines are Hek293 and CAP cells, both of which originate from human amniocytes. Because of higher yields of active FVIII molecules CAP cells, in particular, CAP-T cells were chosen as the preferred expression system for transient transfection and CAP-Go cells for stable expression.

**[0138]** Transient transfection was performed with nucleofection programs. The supernatants were screened for FVIII activity and antigen. Purification of the recombinant proteins from CAP cells was done, including FVIII affinity chromatography.

**[0139]** In detail, CAP-T cells (Cevec Pharmaceuticals, Köln, Germany) were cultured in PEM medium supplemented with 4 mM GlutaMAX (Thermo Fisher Scientific, 35050038) and 5 µg/ml blasticidin (Thermo Fisher Scientific, R21001; complete PEM medium). In order to thaw the cells, the required amount of frozen vials were transferred to a 37 °C water bath. After thawing, each vial was transferred to 10 ml of chilled, complete PEM medium. The cell suspension was centrifuged at 150 x g for 5 minutes. During this washing step the dimethyl sulfoxide (DMSO) used for cryopreservation was removed. The pellet was resuspended in 15 ml warm, complete PEM medium and transferred to a 125 ml shaker flask. The cells were incubated at 37 °C in a humidified incubator with an atmosphere containing 5 % CO<sub>2</sub>. The flasks were set on a shaking platform, rotating at 185 rpm with an orbit of 50 mm. Subculturing of the cells was performed every 3 to 4 days. The fresh culture was set to 0.5x10<sup>6</sup> cells/ml by transferring the required amount of cultured cell suspension to a new flask and adding complete PEM medium. In the case that the transferred cell suspension would exceed 20% of the total volume, the suspension was centrifuged at 150 x g for 5 minutes and the pellet was resuspended in fresh complete PEM medium. The volume of cell suspension per shaking flask was 20% of the total flask volume.

**[0140]** A minimum of three subcultures were performed after thawing before transfection experiments were performed.

#### Protein expression in CAP-T cells by transient transfection

**[0141]** The CAP-T cells were transfected using the 4D-Nucleofector™ (Lonza, Basel, Switzerland). For each transfection 10x10<sup>6</sup> CAP-T cells were centrifuged at 150 x g for 5 minutes in 15 ml conical tubes. The cells were resuspended in 95 µl supplemented SE Buffer, taking into account the volume of the pellet and the volume of the plasmid solution. Afterwards, 5 µg of the respective plasmid were added to the cell suspension followed by gentle mixing. The solution was transferred to 100 µl Nucleocuvettes. The used transfection program was ED-100. After the transfection, the cells from one Nucleocuvette were transferred to 125 ml shaker flasks, containing 12.5 ml complete PEM medium. The cells were cultivated for 4 days as described above. At day 4 the cells were harvested by centrifugation at 150 x g for 5 minutes. Larger protein amounts could be produced by combining 12.5 ml approaches as described above.

**[0142]** Supernatants were screened for FVIII activity and antigen directly after harvest.

**[0143]** The recombinant Factor VIII protein was further analyzed. FVIII activity was measured by chromogenic activity assay and clotting activity FSL assay. The antigen was estimated by FVIII antigen ELISA. As a further assay for biological activity, the cleavage of the recombinant proteins by thrombin was analyzed. Moreover, chain distribution and appearance was tested by Western Blots. Further, vWF-binding and albumin binding were tested.

#### FVIII activity - chromogenic activity assay

**[0144]** The activity of FVIII was determined by a chromogenic assay. In this two-step assay, FIXa and FVIIIa activate



FX in the first step. In the second step, the activated FX hydrolyses a chromogenic substrate, resulting in a color change, which can be measured at 405 nm. Due to the fact that calcium and phospholipids are present in optimal amounts and an excess of FIXa and FX is available, the activation rate of FX is only dependent on the amount of active FVIII in the sample.

**[0145]** The reagents for this chromogenic FVIII activity assay were taken from the Coatest® SP FVIII Kit. The kit contained phospholipids, calcium chloride (CaCl<sub>2</sub>), trace amounts of thrombin, the substrate S-2765, a mixture of FIXa and FX and the thrombin inhibitor I-2581. The inhibitor was added, in order to prevent hydrolysis of the substrate by thrombin, which was built during the reaction. All dilutions were performed in distilled water or Tris-BSA (TBSA) Buffer, containing 25 mM Tris, 150 mM sodium chloride (NaCl) and 1 % Bovine serum albumin (BSA), set to pH 7.4. Each sample was diluted at least 1:2 with FVIII-depleted plasma. Further dilutions were performed using the TBSA Buffer.

**[0146]** The assay was performed using the BCS XP (Siemens Healthcare, Erlangen, Germany), a fully automated hemostasis analyzer. All reagents including water, TBSA Buffer and the samples were inserted into the analyzer. For each sample the analyzer mixed 34 µl calcium chloride, 20 µl TBSA Buffer, 10 µl sample, 40 µl water, 11 µl phospholipids and 56 µl FIXa-FX-mixture. This mixture was incubated for 300 seconds. Afterwards, 50 µl of S-2765 + I-2581 were added to the reaction. Upon addition of the substrate, the absorption at 405 nm was measured for 200 seconds.

**[0147]** In order to calculate the amount of active FVIII, the software of the analyzer evaluated the slope of the measured kinetic between 30 seconds and 190 seconds after starting the reaction. This result was correlated to a calibration curve, generated with a biological reference preparation (BRP) of FVIII. The activity of the BRP is indicated in IU/ml. However, IU/ml can be assumed equivalent to U/ml. The results were indicated as "% of normal". These results were converted to U/ml, as 100 % of normal FVIII activity are equivalent to 1 U FVIII activity per ml.

#### Clotting Activity FSL

**[0148]** In addition to the two-stage chromogenic assay (see above), a one-stage clotting assay was also performed in order to determine the amount of active FVIII. During this assay, FVIII-depleted plasma, CaCl<sub>2</sub>, the activator Actin FSL and the FVIII-containing sample are mixed in one step. The activator leads to the generation of FIXa, which activates FIX. FVIIIa, FIXa and FX built the tenase complex and FX becomes activated. Further activation of prothrombin and fibrinogen finally leads to the formation of a fibrin clot. The time needed to form the clot, the activated partial thromboplastin time (aPTT), is measured. The aPTT varies, depending on the amount of FVIII.

**[0149]** The clotting assay was performed using the BCS XP. TBSA Buffer, FVIII-depleted plasma, Actin FSL, CaCl<sub>2</sub> and the sample were inserted into the analyzer. The sample was diluted at least 1:2 with FVIII-depleted plasma. Further dilutions were performed using the TBSA Buffer. For each sample the analyzer mixed 45 µl TBSA Buffer, 5 µl sample, 50 µl FVIII-depleted plasma and 50 µl Actin FSL. The reaction was started by the addition of 50 µl CaCl<sub>2</sub>. The analyzer measured the time needed for clot formation.

**[0150]** In order to calculate the amount of active FVIII, the software of the analyzer evaluated a baseline extinction at 405 nm at the beginning of the reaction. All of the following extinction values, within a time of 200 seconds, were analysed regarding their difference to the baseline extinction. The first time point exceeding a defined threshold was determined as the clotting time. This result was correlated to a calibration curve, generated with a BRP of FVIII.

#### FVIII antigen ELISA

**[0151]** The amount of FVIII antigen was determined using the Asserachrom® VIII:Ag ELISA (Diagnostica Stago, Asnières sur Seine Cedex, France). In this sandwich ELISA, the applied FVIII is bound by mouse monoclonal anti-human FVIII F(ab')<sub>2</sub> fragments, which are coated to the plate by the manufacturer. The detection of the bound FVIII occurs via mouse monoclonal anti-human FVIII antibodies, which are coupled to a peroxidase. In the case that FVIII is present, the peroxidase-coupled antibody binds to FVIII and can be detected by the addition of a tetramethylbenzidine (TMB) solution. TMB turns from a clear to a blue-green solution upon reaction with peroxidase. After a short time, this reaction is stopped by the addition of sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), which turns the solution yellow. The amount of bound FVIII correlates with the intensity of the yellow color, which can be measured at 450 nm. The final amounts of FVIII are calculated using a calibration curve generated by the measurement of at least five serial dilutions of a calibrator with a known antigen concentration.

**[0152]** The supplied calibrator and control were reconstituted with 500 µl of distilled water, 30 minutes before starting the ELISA. After this incubation time, the calibrator was diluted 1:10 in the supplied phosphate buffer. This represented the starting concentration. The calibrator was further serially diluted 1:2 up to a dilution of 1:64. As the concentration of the calibrator contained approximately 1 U/ml FVIII, depending on the batch, the starting concentration was equivalent to 0.1 U/ml FVIII whereas the last dilution contained approximately 0.0016 U/ml FVIII. The control was diluted 1:10 and 1:20 with the phosphate buffer. All samples were diluted with the phosphate buffer, depending on their previously determined activity (see above) with the aim to be in the middle of the calibration curve. After the dilution of FVIII samples,

control and calibrator, 200  $\mu$ l of each solution were applied per well in duplicates. In addition to that, two wells were filled with 200  $\mu$ l of phosphate buffer as a blank control. The plate was incubated for 2 hours at room temperature covered with a film. During this time, the peroxidase-coupled anti-human FVIII antibodies were reconstituted with 8 ml phosphate buffer and incubated 30 minutes at room temperature. After the antigen immobilization, the wells were washed five times with the supplied washing solution, which was previously diluted 1:20 with distilled water. Immediately after the washing, 200  $\mu$ l of the peroxidase-coupled anti-human FVIII antibodies were added to each well and incubated for 2 hours at room temperature covered by a film. Afterwards, the plate was washed five times as before. In order to reveal the amount of bound FVIII, 200  $\mu$ l of TMB solution were added to each well and incubated for exactly 5 minutes at room temperature. This reaction was stopped by the addition of 50  $\mu$ l 1 M  $H_2SO_4$  to each well. After an incubation time of 15 minutes at room temperature, the absorbance of each well was measured at 450 nm using the POLARstar Omega plate reader (BMG LABTECH, Ortenberg, Germany).

**[0153]** The results of the ELISA were calculated using the MARS software (BMG Labtech). In a first step, all wells were blank corrected and the mean of the duplicates was calculated. Afterwards, a 4-parameter fit was applied, in order to calculate the concentrations from the calibration curve. According to this calibration curve the amount of FVIII antigen in each well was determined. In the last step, the values were corrected by the dilution factor, resulting in the FVIII antigen amount of each sample.

#### Albumin binding capacity assay

**[0154]** 20% human serum albumin (HSA) was diluted 1:4000 in PBS. 96-well ELISA plates were filled with 100  $\mu$ l/well with diluted HSA solution and coated during a 2 h incubation at 37°C and 400 rpm on a thermoshaker. ELISA plates were washed 3-times with 300  $\mu$ l/well washing buffer. Standard control and FVIII samples either with or without Albumin pre-incubation were diluted with Tris/NaCl pH 7.4 to a concentration of 0.5 U/ml chromogenic activity and 100  $\mu$ l/well were added as 7-step 1:2 serial dilution. Incubation was performed for 1 h at 37°C covered on a thermoshaker. In the meantime, FIXa and FX were resolved together in 10 ml aqua dest., substrate (S-2765 and I-2581) was solved in 12 ml aqua dest.. After FVIII incubation, plates were washed again 3 times with 300  $\mu$ l/well washing buffer. Phospholipides and the FIXa/FX solution were mixed 1:5 and subsequently 50  $\mu$ l/well of this solution were added and incubated for 5 min at 37°C. Without any washing step 25  $\mu$ l  $CaCl_2$  was added to each well, followed by 5 min incubation at 37°C. Finally, 50  $\mu$ l/well substrate were added and detection of activated FX-mediated substrate turnover was performed at 405 nm for 25 cycles followed by end point measurement using an ELISA reader.

#### vWF binding capacity assay

**[0155]** 1 U/ml of each FVIII molecule was either pre-incubated or not with 40 mg/ml albumin for 30 min at RT to promote ABD-albumin binding and an assay for determining the vWF binding capacity was performed as follows: Plasma purified vWF (Biotest AG) was diluted with 0.9% NaCl solution to a concentration of 0.1 U/ml. Coating onto 96-well ELISA plates was done by transferring 100  $\mu$ l of this solution to each well followed by an 2 h incubation at 37°C and 400 rpm. The wells were washed 3 times with 300  $\mu$ l of washing buffer (8 mM sodium phosphate, 2 mM potassium phosphate, 0.14M NaCl, 10 mM KCl, 0.05% Tween-20, pH 7.4). FVIII standard (commercial rFVIII without vWF) and samples were pre-diluted with dilution buffer (25 mM Tris, 150 mM NaCl, pH 7.4) to a concentration of 0.25 U/ml according to chromogenic activity and transferred as a 7-step, serial 1:2 dilution into each plate well (100  $\mu$ l/well). Incubation was carried out for 1 h at 37°C and 400 rpm. In the meantime, FIXa and FX were resolved together in 10 ml aqua dest., substrate (S-2765 and I-2581) was solved in 12 ml aqua dest.. After FVIII incubation, plates were washed again 3 times with 300  $\mu$ l/well washing buffer. Phospholipides and the FIXa/FX solution were mixed 1:5 and subsequently 50  $\mu$ l/well of this solution were added and incubated for 5 min at 37°C. Without any washing step 25  $\mu$ l  $CaCl_2$  was added to each well, followed by 5 min incubation at 37°C. Finally, 50  $\mu$ l/well substrate were added and detection of activated FX-mediated substrate turnover was performed at 405 nm for 25 cycles followed by end point measurement using an ELISA reader.

#### Western Blot

##### Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

**[0156]** Cell supernatants, cell lysates or purified material of FVIII variants were appropriately diluted with 1x NuPAGE LDS Sample Buffer (4x, Thermo Fisher Scientific, NP0007) and further diluted 1:2 with reducing sample buffer. Reducing sample buffer was produced by combining 2.5 parts of NuPAGE LDS Sample Buffer with 1 part of NuPAGE Sample Reducing Agent (10x, Thermo Fisher Scientific, NP0004). 20  $\mu$ l of each sample were mixed with 20  $\mu$ l of reducing sample buffer in a 1.5 ml vial and heated for 10 min at 70°C using a thermoshaker (Eppendorf). A NuPAGE 4-12% Bis-

Tris Protein Gel (Thermo Fisher Scientific) was inserted into the XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific) and inner and outer chambers were filled with 1x NuPAGE MOPS SDS Running buffer (Thermo Fisher Scientific, NP0001). 500  $\mu$ l of NuPAGE Antioxidant (Thermo Fisher Scientific) was added to the inner chamber. 10  $\mu$ l of the each prepared sample and 4  $\mu$ l of Precision Plus Protein All Blue Standard (Bio-Rad, 161-0373) diluted 1/10 in 1x LDS Sample Buffer were loaded onto the gel. The sample separation was achieved by running the gel at a constant voltage of 200 V for 50-60 min.

#### Non-reducing SDS-PAGE

**[0157]** 20  $\mu$ l of cell supernatants, cell lysates or purified material of FVIII variants were, either pre-diluted or not, appropriately diluted with 10  $\mu$ l of NuPAGE LDS Sample Buffer (4x, Thermo Fisher Scientific, NP0007) and 10  $\mu$ l aqua dest.. Samples were heated for 10 min at 70°C using a thermoshaker (Eppendorf). A NuPAGE™ 3-8% Tris-Acetate Protein Gel (Thermo Fisher Scientific) was inserted into the XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific) and inner and outer chambers were filled with 1x NuPAGE™ Tris-Acetate SDS Running Buffer (Thermo Fisher Scientific, LA0041). 10  $\mu$ l of the each prepared sample and 4  $\mu$ l of Precision Plus Protein All Blue Standard (Bio-Rad, 161-0373) diluted 1/10 in 1x LDS Sample Buffer were loaded onto the gel. The sample separation was achieved by running the gel at a constant voltage of 150 V for 55-70 min.

#### Western Blotting and Detection

**[0158]** To investigate the separated proteins by immunofluorescence detection, they were transferred onto an Odyssey nitrocellulose membrane (Li-Cor) or an Amersham Hybond Low Fluorescence 0.2  $\mu$ m polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Sciences) by using the XCell II blot module (Thermo Fisher Scientific) for semi-wet protein transfer. The PVDF membrane was activated in methanol and then applied to the SDS gel, whereas the nitrocellulose membrane was directly applied to the SDS gel. The system was filled with NuPAGE transfer buffer (20X, Thermo Fisher Scientific) according to the manufacturer's instructions. Protein blotting was performed for 1 h at 30 V. After protein transfer, the membrane was blocked over night at 4°C in Odyssey blocking buffer (Li-Cor). Afterwards the membrane was incubated for 1 h at room temperature simultaneously with either a rabbit anti-coagulation factor VIII monoclonal antibody (Sino Biological, 13909-R226, 1:1000) and a mouse anti-human factor VIII monoclonal antibody (Merck, MAB038, 1:2500) or with 0.0004  $\mu$ g/ $\mu$ l sheep anti-human factor VIII:C polyclonal antibody (Cedarlane, CL20035AP, 1:5000), each diluted in Odyssey Blocking buffer containing 0.05% Tween 20. After incubation, the membrane was washed 4-times for 5 min in 0.1% PBST. For detection of FVIII heavy and light chain the membrane was incubated with 0.067  $\mu$ g/ml IRDye 800CW donkey anti-mouse (Li-Cor, 926-32212, 1:15000) and 0.067  $\mu$ g/ml IRDye 680RD donkey anti-rabbit (Li-Cor, 926-68073, 1:15000) diluted in Odyssey blocking buffer containing 0.05% Tween 20 for 1 h at room temperature. Alternatively, the CF680 donkey anti-sheep IgG (H&L) antibody (Biotium, 20062-1) was used in a 1:5000 dilution in Odyssey blocking buffer for binding the respective primary antibody. Finally, the membrane was washed 4 times for 5 min in 0.1% PBST, 2 times for 5 min in PBS and rinsed in water. The membrane was visualized using the Licor Odyssey Imager.

#### Pharmacodynamic studies

**[0159]** Coagulation factors were administered by a single intravenous tail vein injection into female haemophilia A mice with doses of 200 U/kg body weight or respective amounts of a control solution. After 4 or 20 h post dosing, tail vein transection bleeding assay was performed as follows: The animals were anaesthetised with 5% isoflurane in 30% O<sub>2</sub> and 70% N<sub>2</sub>O, and immediately placed in prone position on a heating pad at +37°C. Tail vein transection was performed as described by Johansen et al., 2016. Haemophilia 22(4):625-631.

**[0160]** Bleeding was monitored for 60 min and bleeding time was determined using a stop clock. Primary bleeding time was noted until first bleeding cessation. After the primary bleeding, the tail was put into a new centrifuge tube filled with pre-warmed saline. If the mouse was not bleeding at 15, 30 and 45 min post injury, the tail was lifted out of the saline and the wound was challenged by gently wiping it twice with a saline wetted gauze swab in the distal direction. Immediately after the challenge, the tail was re-submerged into the saline. The cumulative bleeding time of all following bleeds constitute the secondary bleeding time. The total bleeding time is defined as the sum of the primary and all secondary bleeding times.

**[0161]** Following the determination of bleeding time, the tubes were centrifuged at 4140 g at room temperature for 3 minutes. Apart from 1 mL, the supernatant was removed. The cell pellet was resuspended and hemoglobin content was determined by using a method similar to that described by Elm et al. (2012).

## Results and Discussion

[0162] Generation and prescreening of several different FVIII-ABD fusion proteins covering FVIII double chain and single chain constructs was very promising in initial experiments and similar in both single chain and double chain backbones.

[0163] The formation of single chain FVIII molecule was increased compared to double chain forms when ABD was fused in between heavy chain and light chain (data not shown).

[0164] The FVIII proteins further developed and produced shown herein are listed in Table 1.

**Table 1 Structure of relevant variants of FVIII with ABD fusion.** A= FVIII A1+a1+A2+a2+truncated B domain, C= FVIII a3 (optionally truncated) +A3+C1+C2 domains, L= Thrombin cleavable linker, G= flexible glycine-serine linker 1 (G1), D= ABD2,

Construct name	Structure	Protein of the invention?
AD2CD2_SC	ALDGLGDLCLDGLGD_SC	Yes
ADLCLD_SC	ADLCLD_SC	Yes
AbD2CD2_SC	ALDGLGDLCLDGLGD_SC + Y1699F + Y1683F	Yes
AD2CD_SC	ALDGLGDLCLD_SC	Yes
ACLD_SC	ACLD_SC	No
ADLC_SC	ADLC_SC	No
AD2C_SC	AD2C_SC	No
ACLDGLGD_SC	ACLDGLGD_SC	No
AD2CD2woLG_SC	ADDCDD_SC	Yes
AD2CD2woL_SC	AGDGDGCGDGD_SC	Yes
ACD4woLG_SC	ACDDDD_SC	No
ACL(GD)4_SC	ACLGDDGDGDGD_SC	No

[0165] Six single chain FVIII-ABD fusion molecules were generated *in silico* and respective DNA constructs were tested for their expression in either HEK293 or CAP-T cells (cf. Table 3). As all of those FVIII-ABD variants were expressed, secreted and functional, based on results of the chromogenic FVIII activity measurement, all molecules were produced in midi-scale CAP-T cell culture and successfully purified in larger amounts as needed for further characterizations and PK (pharmacokinetic) analysis.

**Table 3 FVIII-ABD fusion proteins analyzed in supernatants of transfected HEK293 or CAP-T cells.** (n/a: not available)

Construct	Chromogenic activity [U/mL]	Clotting activity [U/mL]	FVIII: Ag [U/mL]	Specific chromogenic activity [%]	Expression Cell line
ADLC_SC	0,98	0,9	0,47	209	HEK293
ACLD_SC	1,26	0,79	0,72	175	HEK293
ADLCLD_SC	1	0,45	n/a	n/a	HEK293
AD2CD2_SC	1,45	0,35	0,71	204	CAP-T
AD2CD_SC	1,3	0,5	0,48	271	CAP-T
AD2C_SC	2,0	1,0	n/a	n/a	CAP-T
ADLC_SC	0,9	n/a	n/a	n/a	CAP-T
ADLCLD_SC	1,9	n/a	n/a	n/a	CAP-T

[0166] All six purified FVIII-ABD fusion variants were extensively characterized by several methods including determination of FVIII antigen and chromogenic activity, Actin FSL clotting, heavy and light chain detection by western blotting (WB), thrombin-cleavage analysis and binding to vWF and albumin. Table 4 gives an overview of produced FVIII-ABD variants in terms of chromogenic and clotting activity as well as antigen levels in the final solutions. Measurement of these values indicated that FVIII-ABD fusion proteins are still capable of their biological function: Bridging factor IXa and factor X leading thereby to the activation of the latter one. Comparison of the specific chromogenic activity (chromogenic activity / antigen \*100) demonstrates that ADLC\_SC and ReFacto AF® are similar (109% vs 104%). However, the specific chromogenic activities of all other FVIII-ABDs are much better, ranging from 130% to 206%.

[0167] Interestingly, the results indicate that increasing numbers of ABD motifs within one FVIII molecule decrease the clotting activity and also the capability of vWF binding. The decrease in clotting activity may be caused by the setup of the assay, which is strictly time-dependent. This may not mirror *in vivo* clotting activity.

**Table 4 Measurements of chromogenic FVIII activity, FSL clotting activity, and antigen levels. The indicated specific activity was calculated by the ratio of chromogenic activity and antigen.**

FVIII-ABD variant	chromogenic activity [U/ml]	FSL clotting [U/ml]	Antigen [U/ml]	Specific activity [%]
ADLC_SC	147	101	135	109
ACLD_SC	150	100	101	149
ADLCLD_SC	128	80	62	206
AD2CD_SC	120	61	92	130
AD2CD2_SC	164	42	96	171
AD2C_SC	112	72	78	144
ReFacto AF® (Control)	253	242	243	104

[0168] Western blot was used to observe the heavy and light chain patterns of the different FVIII-ABD fusion molecules in comparison to ReFacto AF® (data not shown). The analysis demonstrated that, in contrast to ReFacto AF®, the FVIII-ABD variants were mostly expressed as single chain molecules.

[0169] Activation of FVIII-ABD variants was investigated by direct incubation with thrombin at 37°C for 8 min and subsequent provision for reducing SDS-PAGE followed by western blotting. Band patterns of thrombin-activated or untreated FVIII-ABD molecules show that all FVIII-ABD molecules were activated by Thrombin in a comparable manner as ReFacto AF® (data not shown).

[0170] Albumin binding of the ADLCLD\_SC variant was tested by an assay in comparison to FVIII 6rs-Ref, demonstrating the capability of albumin binding (Fig 1). An excess of unbound soluble albumin inhibited the binding to plate-bound albumin.

[0171] The influence of ABD and linker modifications on the binding between FVIII and vWF were investigated in two settings: 1. directly, without the presence of albumin; 2. after a 30 min pre-incubation with physiological concentrations of albumin promoting the ABD-albumin binding. As demonstrated in Fig. 2, the vWF binding of FVIII-ABD fusion proteins directly decreases by an increasing number of ABD motifs. However, only one ABD motif per FVIII does not have an influence on the vWF binding in the absence of albumin, independent from its position within the molecule. When FVIII-ABDs were pre-incubated with albumin, a decreased vWF binding was observed for all FVIII-ABD variants. This reduction of vWF binding was higher the more ABD domains were incorporated into FVIII.

[0172] To investigate the impact of linkers on the production and functionality of FVIII-ABD variants, one preferred variant, AD2CD2\_SC, was also produced (I) without any linkers between FVIII and ABD-Domains (AD2CD2woLG\_SC) and (II) with G1 linkers but without thrombin-cleavable L linkers (AD2CD2woL\_SC). These variants were compared to the double chain FVIII 6rs-Ref (ReFacto amino acid sequence), single chain FVIII backbone AC\_SC and two FVIII-ABD variants having four C-terminal ABD domains either without any linkers (ACD4woLG\_SC) or with one thrombin-cleavable linker followed by four ABD domains separated by G1 linkers (ACL(GD)4\_SC).

[0173] Respective plasmids encoding the different FVIII variants were nucleofected into CAP-T cells and 4-day cell culture supernatants were tested for chromogenic FVIII activity, FVIII clotting activity and FVIII antigen levels according to the above-described methods. As shown in Fig. 3, AD2CD2woLG\_SC, ACD4woLG\_SC, and ACL(GD)4\_SC were expressed in only low amounts and chromogenic activity was strongly decreased. AD2CD2woLG\_SC was not expressed in high amounts, but had some specific chromogenic activity. No FVIII clotting activity could be detected for any of these variants. In comparison to all other controls, AD2CD2\_SC and AD2CD2woL\_SC demonstrated good FVIII antigen levels and great FVIII chromogenic and clotting activities, resulting in superior specific chromogenic activity values of approx.

200% or higher. AD2CD2\_SC demonstrate an especially high specific clotting activity.

**[0174]** A western blot analysis based on a non-reduced SDS-PAGE separation of these variants is demonstrated in Fig. 4. Except for FVIII 6rs-Ref, all other variants are mainly present as single chain FVIII molecules. However, AD2CD2woLG\_SC and AD2CD2woL\_SC tend to form multimers or aggregates, which are not observed for the AD2CD2\_SC variant.

### 3. Pharmacokinetic experiments

**[0175]** Purification of FVIII ABD variants was performed for in vivo experiments, based on supernatants of transfected CAP-T cells by strong anion exchange chromatography and affinity chromatography.

**[0176]** To investigate the half-life prolongation effect of ABD motifs introduced into the FVIII molecules, two pharmacokinetic (PK) studies were performed in hemophilia A mice. 12 mice per test item were used, 2 or 3 for each time point. All FVIII-ABD molecules were administered in a single dose of 200 U/kg body weight (6 ml/kg) into the tail vein by a single intravenous tail vein injection into female haemophilia A mice (B6, 129S4-F8<tm1Kaz>/J). Plasma samples taken 0.5, 4, 8, 12, and 20h (and 24h) post injection were analyzed regarding FVIII chromogenic activity and antigen levels in citrate plasma which was subsequently extracted by centrifugation. Plasma samples were stored at -80°C and analyzed for FVIII antigen and chromogenic activity. ReFacto AF® was tested as control beside the FVIII-ABD variants.

**[0177]** Results are shown in Table 5.

**Table 5 Calculated t<sub>1/2</sub> of FVIII-ABD variants.** \*AD2C\_SC data were highly variable. t<sub>1/2</sub> is specified in hours

FVIII-ABD variant	Antigen: t <sub>1/2</sub>	Chromogenic activity: t <sub>1/2</sub>
Study 1		
ACLD_SC	4.8	4.3
AD2CD_SC	4.3	5.0
AD2CD2_SC	14.5	9.4
ReFacto® (control)	5.7	4.1
Study 2		
ADLC_SC	7.8	6.8
ADLCLD_SC	10.4	10.5
AD2C_SC*	13.0	6.1
ReFacto® (control)	7.0	6.8

**[0178]** Thus, by pharmacokinetic studies in hemophilia A mice, preferred FVIII proteins of the invention were identified which show a half-life prolonged up to 2.5x (e.g., ADLCLD\_SC - about 1.5x; AD2CD2\_SC - about 2.5x). Pharmacokinetics of AbD2CD2\_SC were tested in a separate study and were similar to AD2CD2\_SC.

**[0179]** It is noted that the hemophilia A mouse model may even underestimate half-life extension due to the discrepancy of murine and human albumin (murine albumin only has a half-life of about two days). Nevertheless, the observed relative extended half-life of the FVIII proteins of the invention already allows a potential reduction of intravenous FVIII injection in hemophilia patients from 2 - 3 days to a once weekly dosing.

**[0180]** Moreover, a pharmacokinetic proof of concept study in albumin-deficient Tg32 mice having a knock-out of murine albumin and expressing human FcRn a-chain instead of the murine one (B6.Cg-Alb<sup>em12Mvw</sup> Fcgrt<sup>tm1Dcr</sup> Tg(FC-GRT)32Dcr/MvwJ) was performed. This mouse model (Alb-/mFcRn-/hFcRn<sup>+</sup>), compared to hemophilia A mice, reveals a situation closer to humans as injected human albumin has a half-life of approx. 20 days which is similar to the half-life in humans. Intravenous FVIII injection (AD2CD2\_SC; ReFacto AF®) was done with 200 U/kg (based on chromogenic activity) plus 1% human albumin.

**[0181]** The results, shown in Fig. 5 demonstrated a half-life extension of AD2CD2\_SC in comparison to ReFacto AF® of about 4x, allowing a potential reduction in patients of i.v. FVIII injection from 2 - 3 days to a 8 - 12 days dosing.

**[0182]** In addition, a pharmacokinetic study was performed in Göttingen Minipigs. Three animals per group were injected with 30 U FVIII antigen/kg body weight with either (I) ReFacto AF + 1% human serum Albumin (HSA), (II) ReFacto AF + 10% HSA, (III) AD2CD2\_SC + 1% HSA or (IV) AD2CD2\_SC + 10% HSA via the ear vein. Blood samples were taken predose, 4, 12, 36, 48, and 120 h post administration and citrate plasma was isolated immediately by centrifugation. Bioanalytical sample measurement was performed by FVIII antigen ELISA, which is specific for human

FVIII and does not detect any porcine FVIII. Evaluation by non-compartment analysis (Figure 6) obtained half-lives for (I) ReFacto AF + 1% HSA of 7.1 h, (II) ReFacto AF + 10% HSA of 6.4 h, (III) AD2CD2\_SC + 1% HSA of 18.6 h, and (IV) AD2CD2\_SC + 10% HSA of 20.7 h. Thus, an half-life extension of approx. 3-fold was observed for AD2CD2\_SC compared to ReFacto AF in this model.

**[0183]** Moreover, pharmacodynamics studies have been performed. Hemophilia A mice (Jackson No. B6; 129S4-F8<sup>tm1 Kaz</sup>/J) and control mice (Jackson No. C57BL/6NCrl) were intravenously injected with 200 U/kg (based on chromogenic FVIII activity) of each FVIII variant (ReFacto®, Eloctate®, AD2CD2\_SC, ADLCLD\_SC) or control solutions (Vehicle Control, 0.9% NaCl) and weight loss through bleeding, bleeding time and Hb amount by OD550 have been analyzed. Additional plasma sampling (0.5 h p.a. by retro-orbital withdraw, after experiment) have been done for analysis of FVIII activity. As shown in Fig. 7, all FVIII proteins of the invention diminish total bleeding time and blood loss similar to that of control mice indicating in vivo functionality of AD2CD2\_SC and ADLCLD\_SC.

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## SEQUENCE LISTING

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								1155				1160					1165			
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55					Lys	Lys	His	Thr	Ala	His	Phe	Ser	Lys	Lys	Gly	Glu	Glu	Glu	Asn	Leu
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[illegible]

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			755					760					765			
5	Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Tyr	Asp	Asp	Thr	Ile	Ser	Val	Glu
		770					775					780				
	Met	Lys	Lys	Glu	Asp	Phe	Asp	Ile	Tyr	Asp	Glu	Asp	Glu	Asn	Gln	Ser
	785				790						795					800
	Pro	Arg	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	Tyr	Phe	Ile	Ala	Ala	Val
				805						810					815	
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			820					825						830		
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		850				855						860				
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	865				870						875					880
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Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg  
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Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
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35 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
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His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser  
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40 Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp  
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Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu  
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Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser  
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Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
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Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val  
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	290						295				300					
	Leu	Glu	Ile	Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	Met
	305					310					315					320
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	Trp	Val	His	Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro
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	Ala	Tyr	Thr	Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu
							455					460				
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50	Met	Thr	Ala	Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp
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	Tyr	Tyr	Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys
						740			745					750		
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						755			760				765			
55	Lys	Arg	His	Gln	Arg	Glu	Ile	Thr	Arg	Thr	Thr	Leu	Gln	Ser	Asp	Gln
						770			775			780				
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	Trp	Arg	Val	Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His	Ser
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	Ala	Ser	Gly	His	Ile	Arg	Asp	Phe	Gln	Ile	Thr	Ala	Tyr
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	Leu	Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln	Gln
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	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile	Leu
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	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser	Gly	Ile	Ile
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	Phe	Asn	Pro	Pro	Ile	Ile	Ala	Arg	Tyr	Ile	Arg	Leu	His
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	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	Leu	Met	Leu
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10	Leu	Thr	Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu	Ile	Ser	Ser	Ser	Gln	Asp
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Trp	Asp	Tyr	Met	Gln	Ser	Asp	Leu	Gly	Glu	Leu	Pro	Val	Asp	Ala	Arg	35	40	45	
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Ala	Glu	Val	Tyr	Asp	Thr	Val	Val	Ile	Thr	Leu	Lys	Asn	Met	Ala	Ser	100	105	110	
His	Pro	Val	Ser	Leu	His	Ala	Val	Gly	Val	Ser	Tyr	Trp	Lys	Ala	Ser	115	120	125	
Glu	Gly	Ala	Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp	130	135	140	
Asp	Lys	Val	Phe	Pro	Gly	Gly	Ser	His	Thr	Tyr	Val	Trp	Gln	Val	Leu	145	150	155	160
Lys	Glu	Asn	Gly	Pro	Met	Ala	Ser	Asp	Pro	Leu	Cys	Leu	Thr	Tyr	Ser	165	170	175	
Tyr	Leu	Ser	His	Val	Asp	Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile	180	185	190	
Gly	Ala	Leu	Leu	Val	Cys	Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr	195	200	205	
Gln	Thr	Leu	His	Lys	Phe	Ile	Leu	Leu	Phe	Ala	Val	Phe	Asp	Glu	Gly	210	215	220	
Lys	Ser	Trp	His	Ser	Glu	Thr	Lys	Asn	Ser	Leu	Met	Gln	Asp	Arg	Asp	225	230	235	240
Ala	Ala	Ser	Ala	Arg	Ala	Trp	Pro	Lys	Met	His	Thr	Val	Asn	Gly	Tyr	245	250	255	
Val	Asn	Arg	Ser	Leu	Pro	Gly	Leu	Ile	Gly	Cys	His	Arg	Lys	Ser	Val	260	265	270	
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Phe	Leu	Glu	Gly	His	Thr	Phe	Leu	Val	Arg	Asn	His	Arg	Gln	Ala	Ser	290	295	300	
Leu	Glu	Ile	Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	Met	305	310	315	320
Asp	Leu	Gly	Gln	Phe	Leu	Leu	Phe	Cys	His	Ile	Ser	Ser	His	Gln	His	325	330	335	
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Gln	Leu	Arg	Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp	355	360	365	
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Pro	Ser	Phe	Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr	385	390	395	400
Trp	Val	His	Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro				

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	Glu	Gly	Ala	Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp			
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	Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser					
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	Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile					
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20	Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu					
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Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser  
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Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg  
35 40 45  
50 Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
50 55 60  
Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
65 70 75 80  
Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln  
85 90 95  
55 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
100 105 110

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	His	Pro	Val	Ser	Leu	His	Ala	Val	Gly	Val	Ser	Tyr	Trp	Lys	Ala	Ser
			115					120					125			
	Glu	Gly	Ala	Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp
		130				135						140				
5	Asp	Lys	Val	Phe	Pro	Gly	Gly	Ser	His	Thr	Tyr	Val	Trp	Gln	Val	Leu
	145					150					155					160
	Lys	Glu	Asn	Gly	Pro	Met	Ala	Ser	Asp	Pro	Leu	Cys	Leu	Thr	Tyr	Ser
					165					170					175	
	Tyr	Leu	Ser	His	Val	Asp	Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile
				180					185					190		
10	Gly	Ala	Leu	Leu	Val	Cys	Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr
			195					200					205			
	Gln	Thr	Leu	His	Lys	Phe	Ile	Leu	Leu	Phe	Ala	Val	Phe	Asp	Glu	Gly
		210					215					220				
	Lys	Ser	Trp	His	Ser	Glu	Thr	Lys	Asn	Ser	Leu	Met	Gln	Asp	Arg	Asp
	225					230					235					240
15	Ala	Ala	Ser	Ala	Arg	Ala	Trp	Pro	Lys	Met	His	Thr	Val	Asn	Gly	Tyr
					245					250					255	
	Val	Asn	Arg	Ser	Leu	Pro	Gly	Leu	Ile	Gly	Cys	His	Arg	Lys	Ser	Val
				260					265					270		
	Tyr	Trp	His	Val	Ile	Gly	Met	Gly	Thr	Thr	Pro	Glu	Val	His	Ser	Ile
20				275				280					285			
	Phe	Leu	Glu	Gly	His	Thr	Phe	Leu	Val	Arg	Asn	His	Arg	Gln	Ala	Ser
		290					295					300				
	Leu	Glu	Ile	Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	Met
	305					310					315					320
25	Asp	Leu	Gly	Gln	Phe	Leu	Leu	Phe	Cys	His	Ile	Ser	Ser	His	Gln	His
					325					330					335	
	Asp	Gly	Met	Glu	Ala	Tyr	Val	Lys	Val	Asp	Ser	Cys	Pro	Glu	Glu	Pro
				340					345					350		
	Gln	Leu	Arg	Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp
			355					360					365			
30	Leu	Thr	Asp	Ser	Glu	Met	Asp	Val	Val	Arg	Phe	Asp	Asp	Asp	Asn	Ser
		370					375					380				
	Pro	Ser	Phe	Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr
	385					390					395					400
	Trp	Val	His	Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro
					405					410					415	
35	Leu	Val	Leu	Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn
				420					425					430		
	Asn	Gly	Pro	Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	Met
			435					440					445			
	Ala	Tyr	Thr	Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu
		450					455					460				
40	Ser	Gly	Ile	Leu	Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu
	465					470					475					480
	Leu	Ile	Ile	Phe	Lys	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro
					485					490					495	
	His	Gly	Ile	Thr	Asp	Val	Arg	Pro	Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys
45				500					505					510		
	Gly	Val	Lys	His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe
			515					520					525			
	Lys	Tyr	Lys	Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp
		530					535					540				
50	Pro	Arg	Cys	Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg
	545					550					555					560
	Asp	Leu	Ala	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu
					565					570					575	
	Ser	Val	Asp	Gln	Arg	Gly	Asn	Gln	Ile	Met	Ser	Asp	Lys	Arg	Asn	Val
				580					585					590		
55	Ile	Leu	Phe	Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu
			595					600					605			
	Asn	Ile	Gln	Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp

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[illegible]

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	Tyr	Arg	Phe	His	Ala	Ile	Asn	Gly	Tyr	Ile	Met	Asp	Thr	Leu	Pro	Gly	
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	Leu	Val	Met	Ala	Gln	Asp	Gln	Arg	Ile	Arg	Trp	Tyr	Leu	Leu	Ser	Met	
					1140					1145					1150		
5	Gly	Ser	Asn	Glu	Asn	Ile	His	Ser	Ile	His	Phe	Ser	Gly	His	Val	Phe	
					1155				1160					1165			
	Thr	Val	Arg	Lys	Lys	Glu	Glu	Tyr	Lys	Met	Ala	Leu	Tyr	Asn	Leu	Tyr	
					1170				1175					1180			
	Pro	Gly	Val	Phe	Glu	Thr	Val	Glu	Met	Leu	Pro	Ser	Lys	Ala	Gly	Ile	
					1185				1190				1195			1200	
10	Trp	Arg	Val	Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His	Ala	Gly	Met	Ser	
					1205					1210					1215		
	Thr	Leu	Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro	Leu	Gly	Met	
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	Ala	Ser	Gly	His	Ile	Arg	Asp	Phe	Gln	Ile	Thr	Ala	Ser	Gly	Gln	Tyr	
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15	Gly	Gln	Trp	Ala	Pro	Lys	Leu	Ala	Arg	Leu	His	Tyr	Ser	Gly	Ser	Ile	
					1250				1255				1260				
	Asn	Ala	Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	Trp	Ile	Lys	Val	Asp	Leu	
					1265				1270			1275				1280	
	Leu	Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln	Gly	Ala	Arg	Gln	
					1285					1290					1295		
20	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile	Met	Tyr	Ser	Leu	
					1300				1305					1310			
	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	Asn	Ser	Thr	Gly	Thr	Leu	
					1315				1320					1325			
	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser	Gly	Ile	Lys	His	Asn	Ile	
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	Phe	Asn	Pro	Pro	Ile	Ile	Ala	Arg	Tyr	Ile	Arg	Leu	His	Pro	Thr	His	
					1345				1350			1355				1360	
	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	Leu	Met	Gly	Cys	Asp	Leu	
					1365				1370						1375		
30	Asn	Ser	Cys	Ser	Met	Pro	Leu	Gly	Met	Glu	Ser	Lys	Ala	Ile	Ser	Asp	
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	Ala	Gln	Ile	Thr	Ala	Ser	Ser	Tyr	Phe	Thr	Asn	Met	Phe	Ala	Thr	Trp	
					1395				1400					1405			
	Ser	Pro	Ser	Lys	Ala	Arg	Leu	His	Leu	Gln	Gly	Arg	Ser	Asn	Ala	Trp	
					1410				1415				1420				
35	Arg	Pro	Gln	Val	Asn	Asn	Pro	Lys	Glu	Trp	Leu	Gln	Val	Asp	Phe	Gln	
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	Lys	Thr	Met	Lys	Val	Thr	Gly	Val	Thr	Thr	Gln	Gly	Val	Lys	Ser	Leu	
					1445					1450					1455		
	Leu	Thr	Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu	Ile	Ser	Ser	Ser	Gln	Asp	
					1460				1465						1470		
40	Gly	His	Gln	Trp	Thr	Leu	Phe	Phe	Gln	Asn	Gly	Lys	Val	Lys	Val	Phe	
					1475				1480					1485			
	Gln	Gly	Asn	Gln	Asp	Ser	Phe	Thr	Pro	Val	Val	Asn	Ser	Leu	Asp	Pro	
					1490				1495				1500				
	Pro	Leu	Leu	Thr	Arg	Tyr	Leu	Arg	Ile	His	Pro	Gln	Ser	Trp	Val	His	
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	Gln	Ile	Ala	Leu	Arg	Met	Glu	Val	Leu	Gly	Cys	Glu	Ala	Gln	Asp	Leu	
					1525					1530					1535		
	Tyr	Leu	Ala	Glu	Ala	Lys	Glu	Ala	Ala	Asn	Ala	Glu	Leu	Asp	Ser	Tyr	
					1540				1545					1550			
50	Gly	Val	Ser	Asp	Phe	Tyr	Lys	Arg	Leu	Ile	Asp	Lys	Ala	Lys	Thr	Val	
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	Glu	Gly	Val	Glu	Ala	Leu	Lys	Asp	Ala	Ile	Leu	Ala	Ala	Leu	Pro	Leu	
					1570				1575				1580				
	Ala	Glu	Ala	Lys	Glu	Ala	Ala	Asn	Ala	Glu	Leu	Asp	Ser	Tyr	Gly	Val	
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55	Ser	Asp	Phe	Tyr	Lys	Arg	Leu	Ile	Asp	Lys	Ala	Lys	Thr	Val	Glu	Gly	
					1605				1610						1615		
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1620

1625

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Trp	Asp	Tyr	Met	Gln	Ser	Asp	Leu	Gly	Glu	Leu	Pro	Val	Asp	Ala	Arg	35	40	45	
Phe	Pro	Pro	Arg	Val	Pro	Lys	Ser	Phe	Pro	Phe	Asn	Thr	Ser	Val	Val	50	55	60	
Tyr	Lys	Lys	Thr	Leu	Phe	Val	Glu	Phe	Thr	Asp	His	Leu	Phe	Asn	Ile	65	70	75	80
Ala	Lys	Pro	Arg	Pro	Pro	Trp	Met	Gly	Leu	Leu	Gly	Pro	Thr	Ile	Gln	85	90	95	
Ala	Glu	Val	Tyr	Asp	Thr	Val	Val	Ile	Thr	Leu	Lys	Asn	Met	Ala	Ser	100	105	110	
His	Pro	Val	Ser	Leu	His	Ala	Val	Gly	Val	Ser	Tyr	Trp	Lys	Ala	Ser	115	120	125	
Glu	Gly	Ala	Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp	130	135	140	
Asp	Lys	Val	Phe	Pro	Gly	Gly	Ser	His	Thr	Tyr	Val	Trp	Gln	Val	Leu	145	150	155	160
Lys	Glu	Asn	Gly	Pro	Met	Ala	Ser	Asp	Pro	Leu	Cys	Leu	Thr	Tyr	Ser	165	170	175	
Tyr	Leu	Ser	His	Val	Asp	Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile	180	185	190	
Gly	Ala	Leu	Leu	Val	Cys	Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr	195	200	205	
Gln	Thr	Leu	His	Lys	Phe	Ile	Leu	Leu	Phe	Ala	Val	Phe	Asp	Glu	Gly	210	215	220	
Lys	Ser	Trp	His	Ser	Glu	Thr	Lys	Asn	Ser	Leu	Met	Gln	Asp	Arg	Asp	225	230	235	240
Ala	Ala	Ser	Ala	Arg	Ala	Trp	Pro	Lys	Met	His	Thr	Val	Asn	Gly	Tyr	245	250	255	
Val	Asn	Arg	Ser	Leu	Pro	Gly	Leu	Ile	Gly	Cys	His	Arg	Lys	Ser	Val	260	265	270	
Tyr	Trp	His	Val	Ile	Gly	Met	Gly	Thr	Thr	Pro	Glu	Val	His	Ser	Ile	275	280	285	
Phe	Leu	Glu	Gly	His	Thr	Phe	Leu	Val	Arg	Asn	His	Arg	Gln	Ala	Ser	290	295	300	
Leu	Glu	Ile	Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	Met	305	310	315	320
Asp	Leu	Gly	Gln	Phe	Leu	Leu	Phe	Cys	His	Ile	Ser	Ser	His	Gln	His	325	330	335	
Asp	Gly	Met	Glu	Ala	Tyr	Val	Lys	Val	Asp	Ser	Cys	Pro	Glu	Glu	Pro	340	345	350	
Gln	Leu	Arg	Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp	355	360	365	
Leu	Thr	Asp	Ser	Glu	Met	Asp	Val	Val	Arg	Phe	Asp	Asp	Asp	Asn	Ser	370	375	380	
Pro	Ser	Phe	Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr	385	390	395	400
Trp	Val	His	Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro				



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					405					410					415	
	Leu	Val	Leu	Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn
				420					425					430		
5	Asn	Gly	Pro	Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	Met
			435					440					445			
	Ala	Tyr	Thr	Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu
	450						455					460				
	Ser	Gly	Ile	Leu	Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu
	465					470					475					480
10	Leu	Ile	Ile	Phe	Lys	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro
				485						490					495	
	His	Gly	Ile	Thr	Asp	Val	Arg	Pro	Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys
				500					505					510		
	Gly	Val	Lys	His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe
			515					520					525			
15	Lys	Tyr	Lys	Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp
	530						535					540				
	Pro	Arg	Cys	Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg
	545					550					555					560
	Asp	Leu	Ala	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu
				565						570					575	
20	Ser	Val	Asp	Gln	Arg	Gly	Asn	Gln	Ile	Met	Ser	Asp	Lys	Arg	Asn	Val
				580					585					590		
	Ile	Leu	Phe	Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu
			595					600					605			
	Asn	Ile	Gln	Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp
	610						615					620				
25	Pro	Glu	Phe	Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val
	625					630					635					640
	Phe	Asp	Ser	Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp
				645					650					655		
	Tyr	Ile	Leu	Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe
30				660					665					670		
	Ser	Gly	Tyr	Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr
		675						680					685			
	Leu	Phe	Pro	Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro
	690						695					700				
	Gly	Leu	Trp	Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly
35	705					710					715					720
	Met	Thr	Ala	Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp
				725						730					735	
	Tyr	Tyr	Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys
				740				745					750			
40	Asn	Asn	Ala	Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Pro	Pro	Val	Leu
			755					760					765			
	Ser	Lys	Asn	Asn	Ala	Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg
		770					775					780				
	His	Pro	Ser	Leu	Ala	Glu	Ala	Lys	Glu	Ala	Ala	Asn	Ala	Glu	Leu	Asp
	785					790					795					800
45	Ser	Tyr	Gly	Val	Ser	Asp	Phe	Tyr	Lys	Arg	Leu	Ile	Asp	Lys	Ala	Lys
				805						810					815	
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				820					825					830		
	Pro	Gly	Ser	Gly	Gly	Ser	Gly	Gly	Ser	Gly	Gly	Ser	Gly	Ser	Gly	Ser
			835				840						845			
50	Lys	Asn	Asn	Ala	Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His
		850					855					860				
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	865					870					875					880
	Ala	Glu	Ala	Lys	Glu	Ala	Ala	Asn	Ala	Glu	Leu	Asp	Ser	Tyr	Gly	Val
				885						890					895	
55	Ser	Asp	Phe	Tyr	Lys	Arg	Leu	Ile	Asp	Lys	Ala	Lys	Thr	Val	Glu	Gly
				900					905					910		

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	Val	Glu	Ala	Leu	Lys	Asp	Ala	Ile	Leu	Ala	Ala	Leu	Pro	Ser	Lys	Asn	
			915					920					925				
	Asn	Ala	Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	Pro	Ser	
		930					935					940					
5	Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Phe	Asp	Asp	Thr	Ile	Ser	Val	Glu	
	945					950					955					960	
	Met	Lys	Lys	Glu	Asp	Phe	Asp	Ile	Phe	Asp	Glu	Asp	Glu	Asn	Gln	Ser	
					965					970					975		
	Pro	Arg	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	Tyr	Phe	Ile	Ala	Ala	Val	
				980					985					990			
10	Glu	Arg	Leu	Trp	Asp	Tyr	Gly	Met	Ser	Ser	Ser	Pro	His	Val	Leu	Arg	
		995						1000					1005				
	Asn	Arg	Ala	Gln	Ser	Gly	Ser	Val	Pro	Gln	Phe	Lys	Lys	Val	Val	Phe	
		1010					1015					1020					
	Gln	Glu	Phe	Thr	Asp	Gly	Ser	Phe	Thr	Gln	Pro	Leu	Tyr	Arg	Gly	Glu	
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	Leu	Asn	Glu	His	Leu	Gly	Leu	Leu	Gly	Pro	Tyr	Ile	Arg	Ala	Glu	Val	
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	Glu	Asp	Asn	Ile	Met	Val	Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	
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	Ser	Phe	Tyr	Ser	Ser	Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	Gln	Gly	
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	Ala	Glu	Pro	Arg	Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	Thr	Tyr	
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	Phe	Trp	Lys	Val	Gln	His	His	Met	Ala	Pro	Thr	Lys	Asp	Glu	Phe	Asp	
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25	Cys	Lys	Ala	Trp	Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu	Lys	Asp	Val	
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	Asn	Pro	Ala	His	Gly	Arg	Gln	Val	Thr	Val	Gln	Glu	Phe	Ala	Leu	Phe	
			1155					1160					1165				
30	Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp	Tyr	Phe	Thr	Glu	Asn	Met	
		1170				1175						1180					
	Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn	Ile	Gln	Met	Glu	Asp	Pro	Thr	
	1185				1190					1195						1200	
	Phe	Lys	Glu	Asn	Tyr	Arg	Phe	His	Ala	Ile	Asn	Gly	Tyr	Ile	Met	Asp	
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35	Thr	Leu	Pro	Gly	Leu	Val	Met	Ala	Gln	Asp	Gln	Arg	Ile	Arg	Trp	Tyr	
				1220					1225					1230			
	Leu	Leu	Ser	Met	Gly	Ser	Asn	Glu	Asn	Ile	His	Ser	Ile	His	Phe	Ser	
			1235					1240					1245				
	Gly	His	Val	Phe	Thr	Val	Arg	Lys	Lys	Glu	Glu	Tyr	Lys	Met	Ala	Leu	
		1250					1255					1260					
40	Tyr	Asn	Leu	Tyr	Pro	Gly	Val	Phe	Glu	Thr	Val	Glu	Met	Leu	Pro	Ser	
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## Claims

1. A recombinant Factor VIII protein comprising a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion, wherein, if the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion.
2. The recombinant Factor VIII protein of claim 1 that is a single chain protein.
3. The recombinant Factor VIII protein of any one of the preceding claims, wherein two albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion.

4. The recombinant Factor VIII protein of any one of the preceding claims, wherein albumin-binding domains are separated from the heavy chain portion and/or the light chain portion and/or other albumin-binding domains by a linker.
5. The recombinant Factor VIII protein of claim 4, wherein the linker comprises a Thrombin-cleavable linker that optionally has the sequence of SEQ ID NO: 39.
6. The recombinant Factor VIII protein of any one claims 4 or 5, wherein the linker comprises a glycine-serine linker that optionally has the sequence of SEQ ID NO: 40 or SEQ ID NO: 41.
7. The recombinant Factor VIII protein of any one claims 4 to 6, wherein said linker comprises a Thrombin-cleavable linker flanked on each side by a glycine-serine linker, wherein said linker optionally has the sequence of SEQ ID NO: 42 or SEQ ID NO: 43.
8. The recombinant Factor VIII protein of any one of the preceding claims, wherein the albumin binding domain comprises a sequence according to SEQ ID NO: 44, wherein preferably the sequence is SEQ ID NO: 46.
9. The recombinant Factor VIII protein of any one of the preceding claims that optionally is a single chain protein, wherein said protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa768 of SEQ ID NO: 16 and a light chain portion having at least 90% sequence identity to aa769-aa1445 of SEQ ID NO: 16.
10. The recombinant Factor VIII protein of any one of the preceding claims that is a single chain protein comprising at least two albumin binding domains between the heavy chain portion and the light chain portion and at least two albumin binding domain C-terminal to the light chain portion, wherein the protein has at least 80% sequence identity to any of SEQ ID NO: 48, 49 or 51, wherein the protein preferably has at least 80% sequence identity to SEQ ID NO: 48.
11. The recombinant Factor VIII protein of any one of the preceding claims, wherein the protein is a de-immunized protein.
12. A nucleic acid encoding a recombinant Factor VIII protein of any one of the preceding claims, wherein said nucleic acid preferably is an expression vector suitable for expression of said recombinant Factor VIII protein in a mammalian cell, optionally, a human cell, such as a CAP cell.
13. A host cell comprising a nucleic acid of claim 12, wherein preferably the host cell is a mammalian cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said cell.
14. A method of preparing a recombinant Factor VIII protein, comprising culturing the host cell of claim 13 under conditions suitable for expression of the Factor VIII protein and isolating the recombinant Factor VIII protein, wherein the method optionally comprises formulating the Factor VIII protein as a pharmaceutical composition.
15. A pharmaceutical composition comprising the recombinant Factor VIII protein of any of claims 1-11, the nucleic acid of claim 12 or the host cell of claim 13, optionally further comprising a biologically acceptable carrier and/or albumin, wherein the pharmaceutical composition preferably is for use in treatment of hemophilia A.

Fig. 1

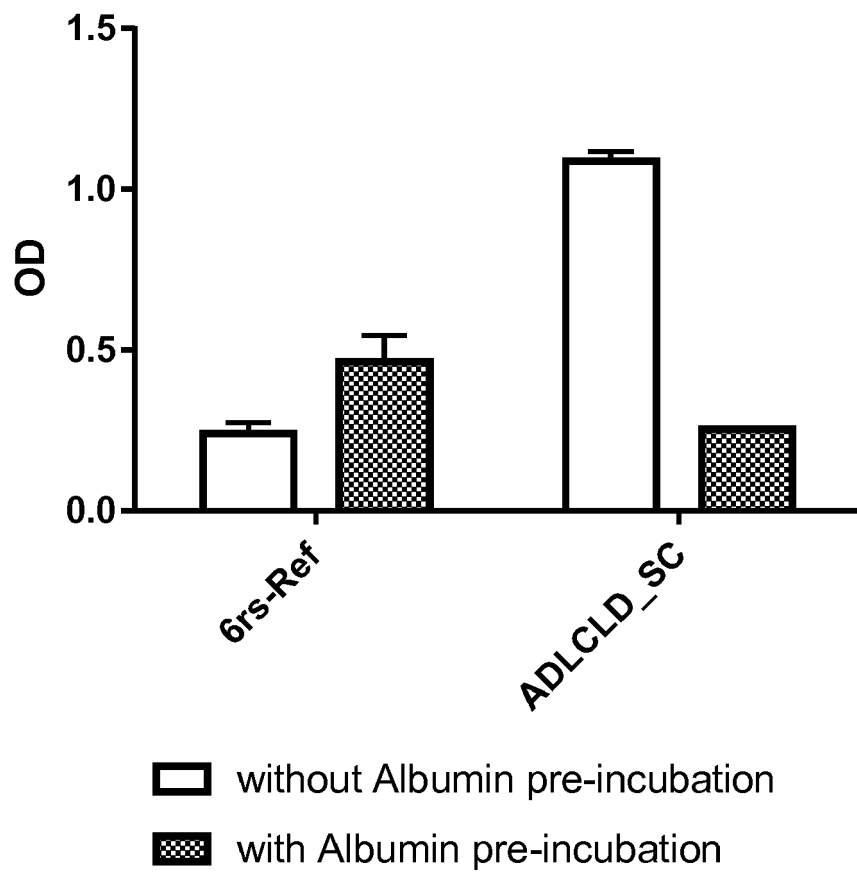
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Fig. 2

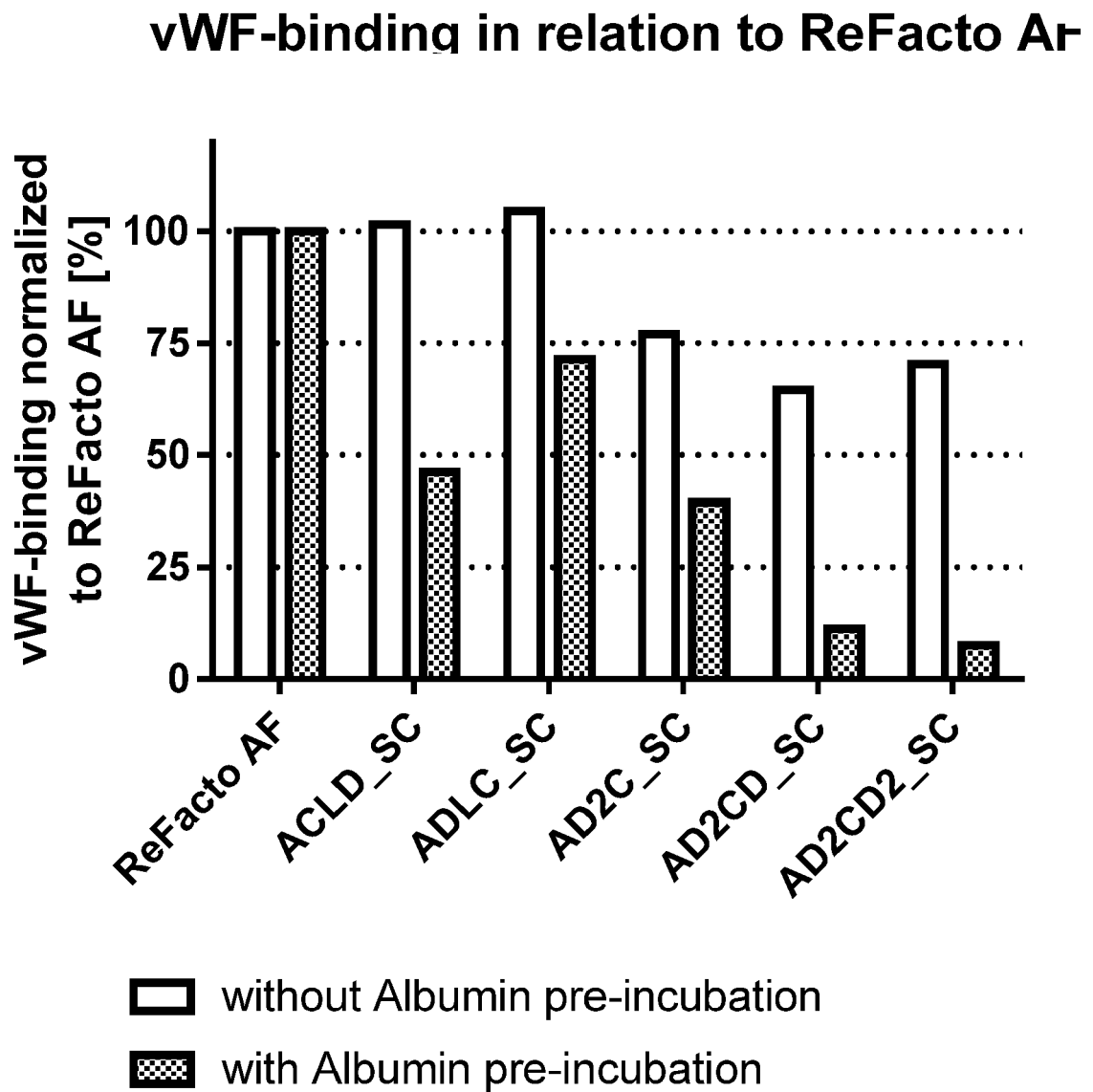


Fig. 3

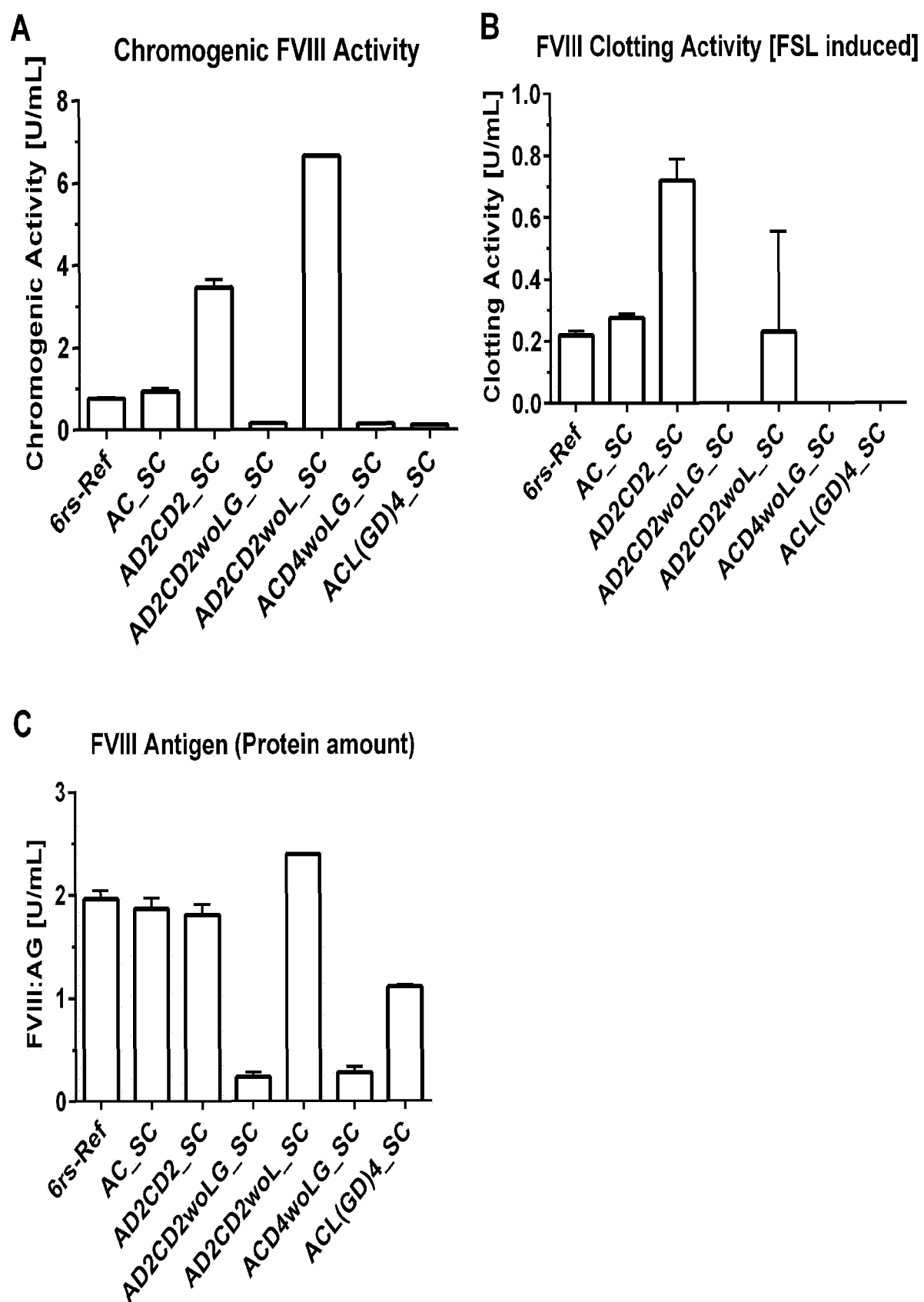


Fig. 3

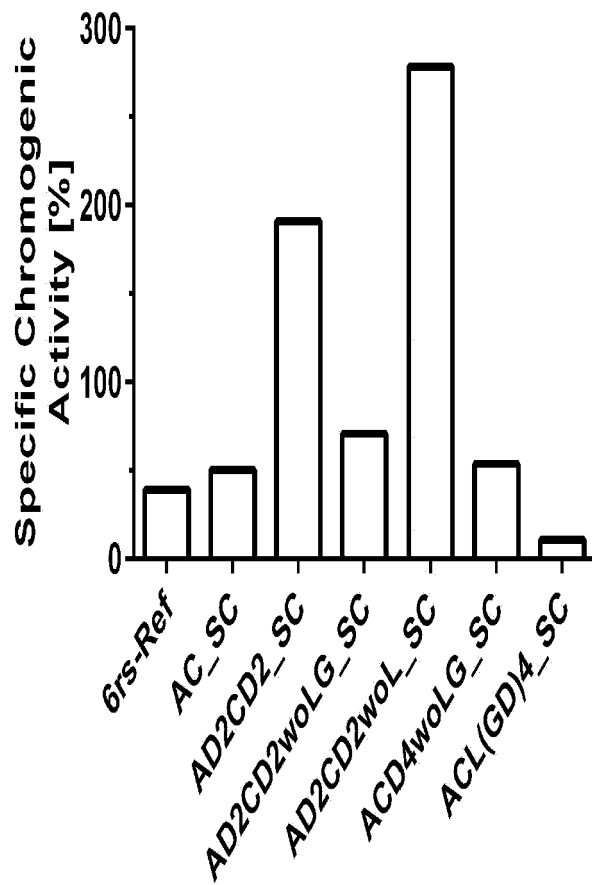
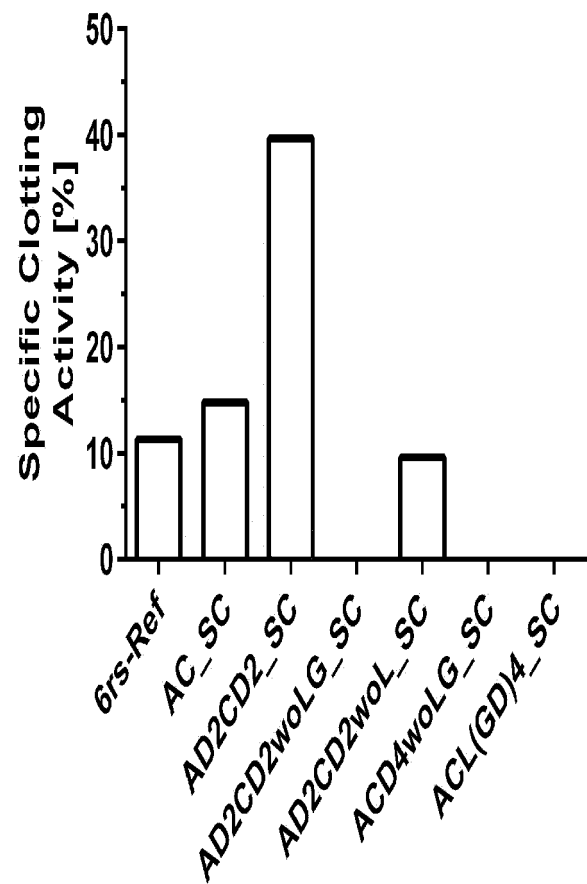
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Fig. 4

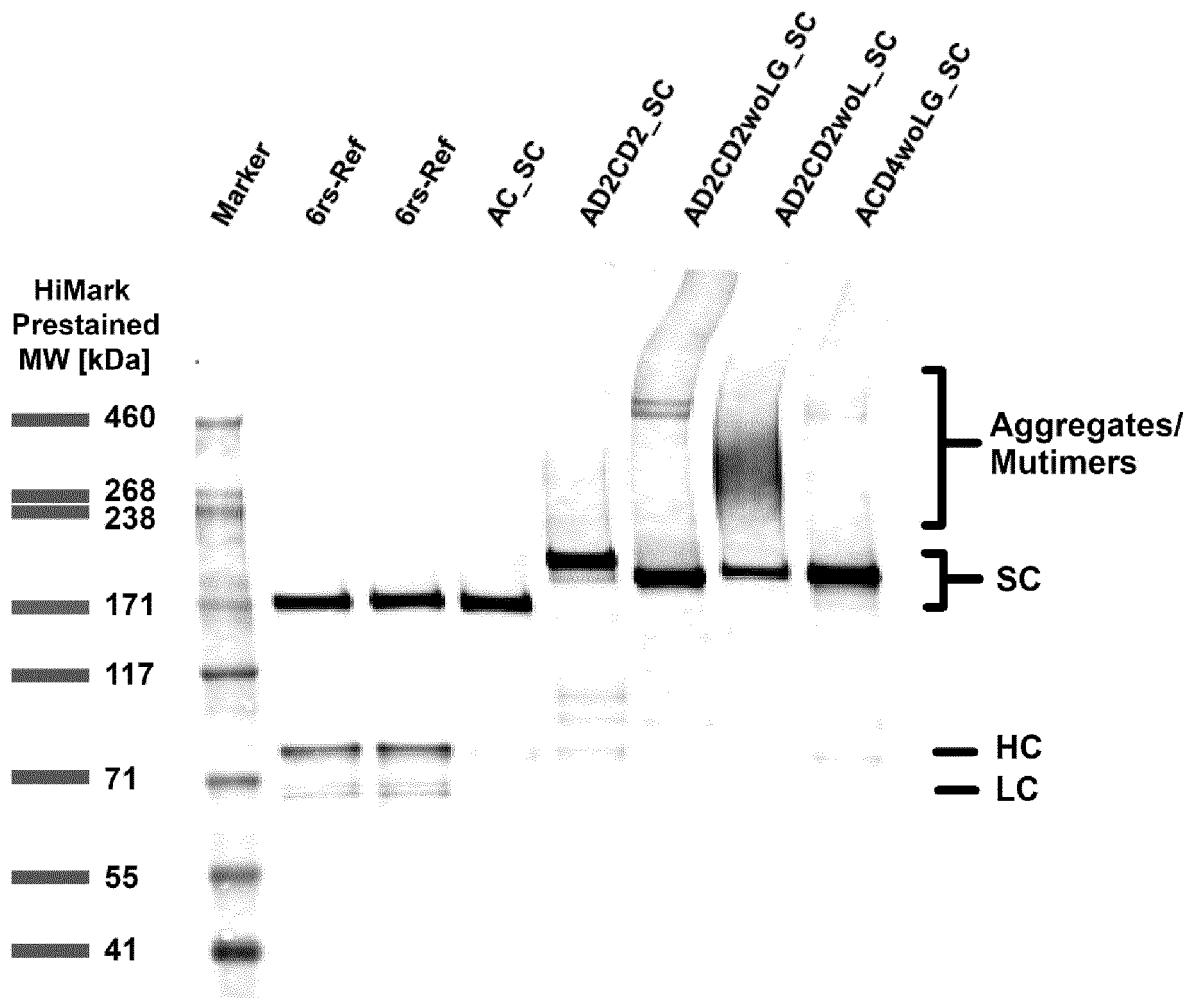




Fig. 5

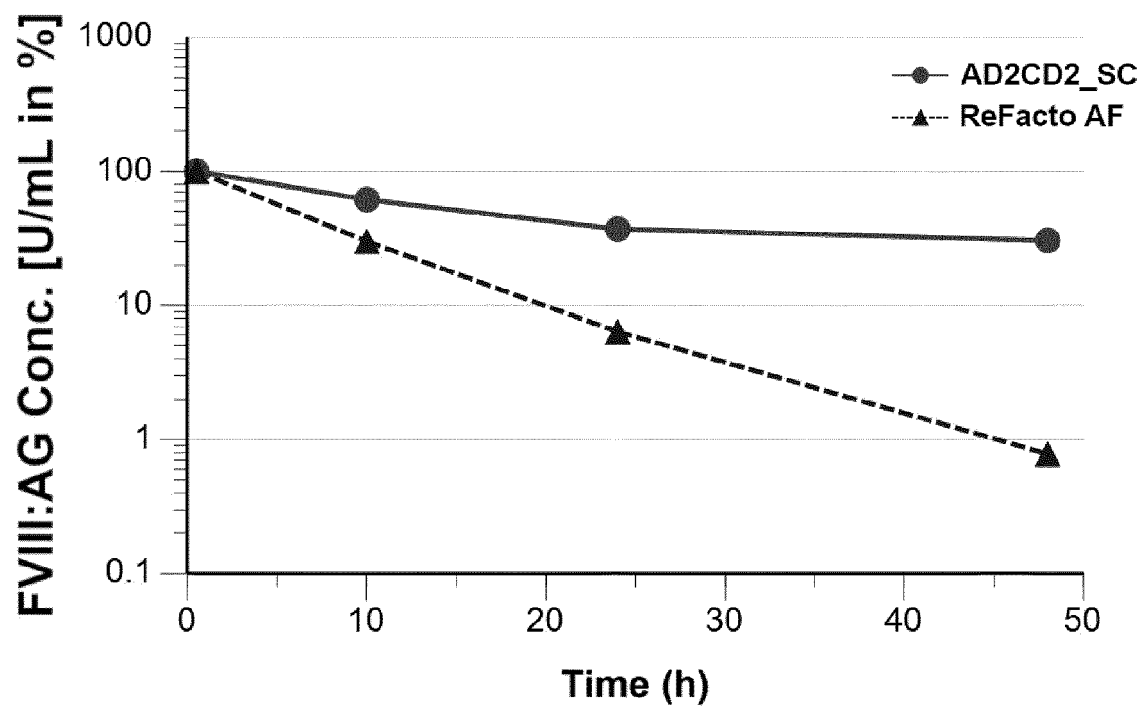


Fig. 6

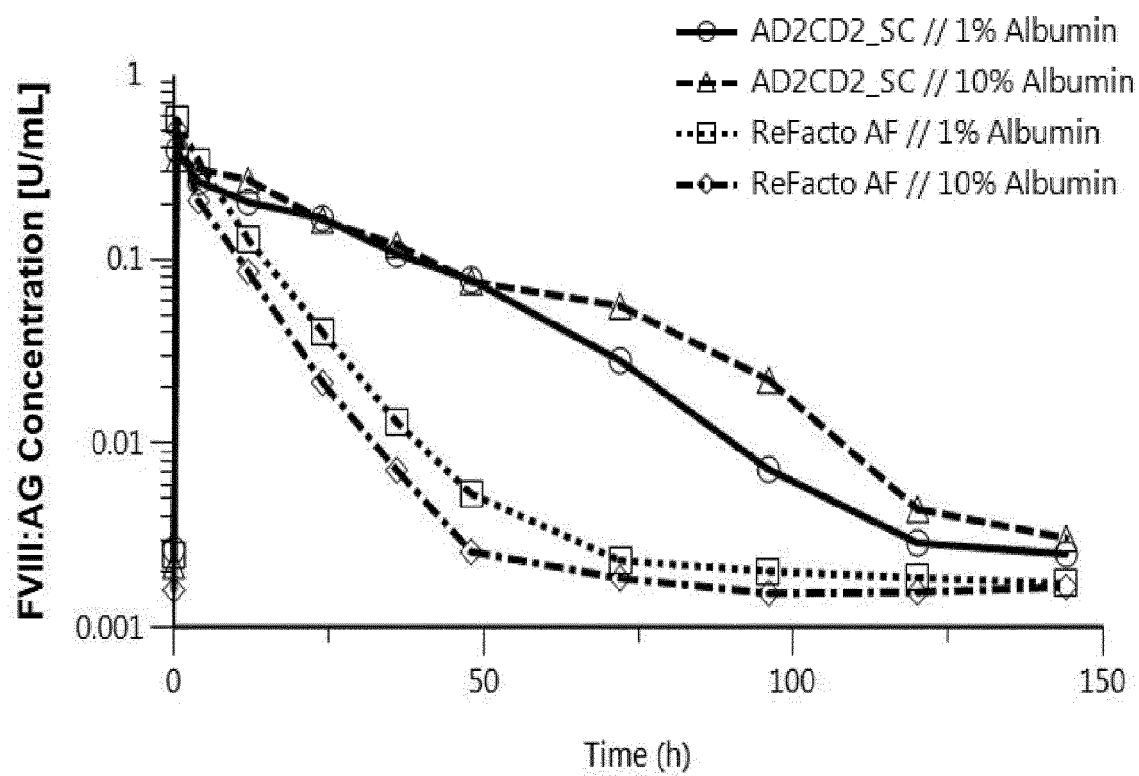
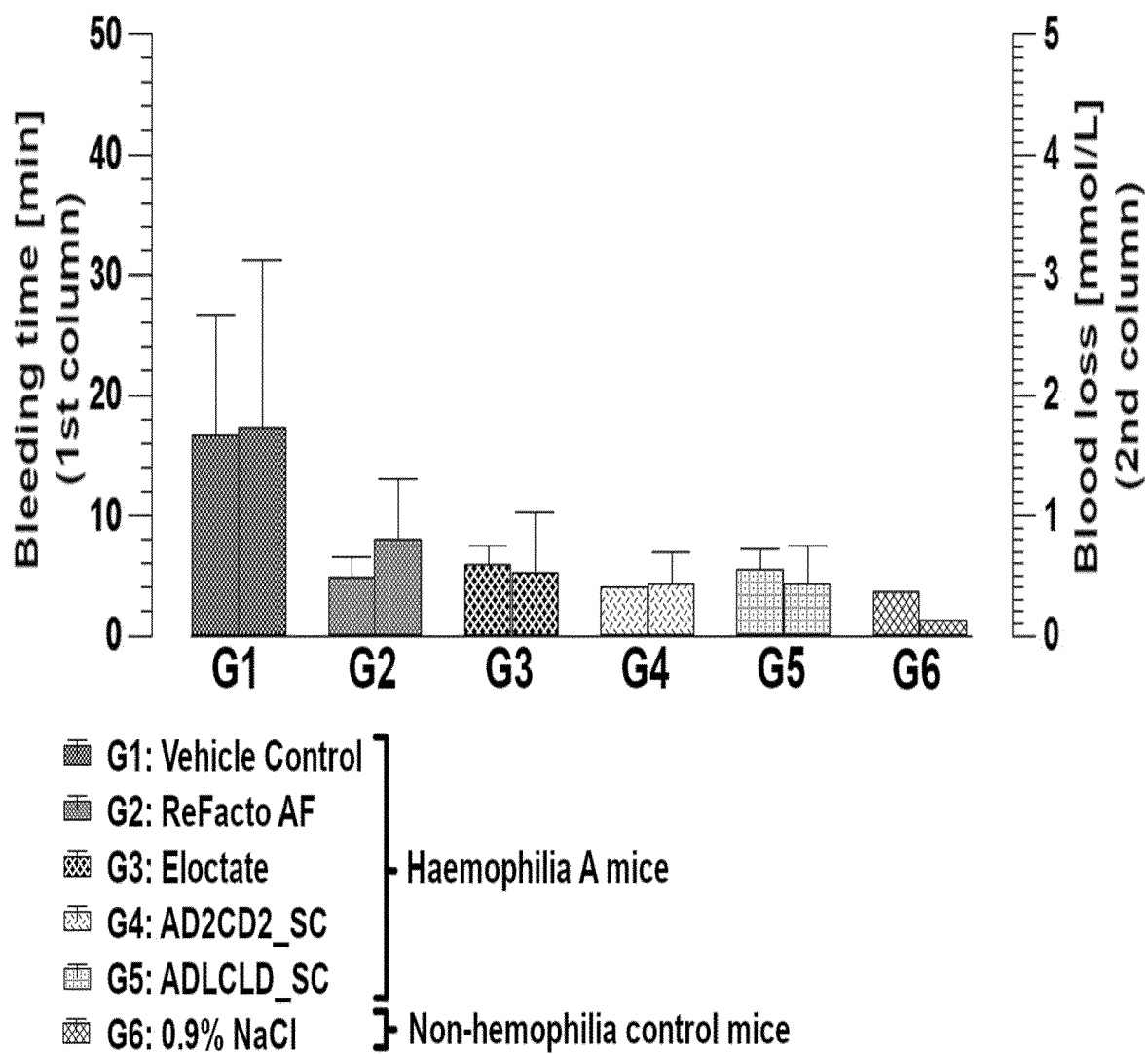


Fig. 7





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X	DANIEL STEINER ET AL: "Half-life extension using serum albumin-binding DARPin domains", PROTEIN ENGINEERING, DESIGN AND SELECTION, vol. 30, no. 9, 17 August 2017 (2017-08-17), pages 583-591, XP55652587, GB ISSN: 1741-0126, DOI: 10.1093/protein/gzx022 * the whole document *	1-15	TECHNICAL FIELDS SEARCHED (IPC) A61K
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 13 December 2019	Examiner Keller, Yves
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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The present search report has been drawn up for all claims			
Place of search <b>Munich</b>		Date of completion of the search <b>13 December 2019</b>	Examiner <b>Keller, Yves</b>
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